REMARKS

In an Office Action dated November 17, 2004, claims 44-51, and 53-64, all of the claims under consideration in the subject patent application, were rejected. By amendment above, claim 46 has been rewritten to correct a typographical error, and claims 47 and 51 have been rewritten. Support for the amendment to claims 47 and 51 can be found in claims 47 and 51 as previously presented.

Reconsideration of this application and allowance of the claims is respectfully requested in view of the following remarks.

The Examiner maintains her objection to the amendment filed December 17, 2003 because she asserts it introduces new matter. According to the Examiner the claim language "wherein the response is measured in step (c) is selected from GTPγS binding" in claims 55 and 60 is not supported by the original disclosure. The Examiner further maintains the rejection of claims 55 and 60 for failing to comply with the written description requirement because the claims contain subject matter which was not described in the specification in such a way as to reasonably convey that the inventors had possession of the claimed invention. The Examiner asserts that the amendments to claim 55, filed December 17, 2003 and claim 60, filed August 25, 2004, are not supported by Examples 14-19 as pointed out in applicants' amendment of December 17, 2003. According to the Examiner the amended claim language is not readily apparent from these sections. In addition, applicants' previous argument was found non-persuasive by the Examiner. Applicants argued that it is generally known in the field of signal transduction that activation through G-protein coupled receptors requires signaling through G-

proteins. Further it is known that G-protein signaling of such receptors requires GTP binding. Therefore, disclosing that the EDG is such a G-protein coupled receptor provides sufficient support for those of skill in the art to understand that measuring GTPγS binding indicates whether the tested material is an agonist or antagonist of the receptor using the assays as described in the disclosure.

Applicants disagree with the Examiner and submit that the assays to determine GTPγS binding are known to those skilled in the art. Further, measuring GTPγS binding is an accepted method in determining agonist/antagonist interaction with G-protein coupled receptors, of which type are the disclosed EDG receptors. See for example the Wieland et al. and Lazareno publications which show that, prior to the filing date of the application, the determination of GTPγS binding is a well accepted method of measuring G-protein coupled receptor activity. (Wieland T., and Jakobs, K., Measurement of Receptor-Stimulated Guanosine 5'-O-(γ-Thio)triphosphate Binding by G proteins; Methods in Enzymology (1994), Vol 237, pp 3-13; and Lazareno, S., Measurement of Agonist-Stimulated [35S]GTPγS Binding to Cell Membranes; Methods in Molecular Biology (1997), Vol 83, pp 107-116; copies attached).

Therefore, Applicants submit that claims 55 and 60 are supported by the disclosure in the specification as under 35 U.S.C 112, first paragraph. Accordingly, withdrawal of the rejection is respectfully requested. In addition, for these reasons no new matter is added to the disclosure and accordingly, withdrawal of the objection to the specification is respectfully requested.

The Examiner also maintained the rejection of claims 44-51 for lack of enablement in the specification for a method of identifying a compound as an agonist for an EDG receptor or a method of identifying a compound as an antagonist for an EDG receptor using the readouts of

NF-kB activation and/or IL-8 production. According to the Examiner the claims are not supported by an enabling disclosure because the specification teaches IL-8 production only in the presence of activated EDG-4 receptor and the NF-kB is taught not to be induced in the presence of activated EDG-1 receptor. Thus, according to the Examiner the recited assays cannot be used to determine agonist/antagonist activity for all types of EDG receptors.

Applicants submit that claims 44-51 were amended to include the limitation that the EDG receptors tested should activate NF-κB or IL-8 or both upon activation of the receptor by an agonist. To further more clearly define the scope of the claims, claim 47 and 51, as amended are directed to only the EDG-4 receptor. Further, applicants disagree with the Examiner's assertions because the claims as amended (including those in applicants' last response) require that the EDG receptors used are those that activate NF-κB and/or IL-8, which does not mean all EDG receptors. Therefore, the Examiner's arguments are misplaced.

Thus, the disclosure is enabling for determining whether a compound is an agonist or antagonist for an EDG receptor by known activation of NF-κB and production of cytokines such as IL-8. Therefore Applicants submit that claims 44-51 are enabled under 35 U.S.C. § 112, first paragraph. Accordingly, withdrawal of the rejection is respectfully requested.

The Examiner also maintained the rejected of claims 53-64 under 35 USC §112, first paragraph, for failing to comply with the enablement requirement. According to the Examiner the specification fails to teach how to identify a compound as an agonist or antagonist of an EDG receptor wherein the activity is measured by modulation of cellular cyclic AMP levels. According to the Examiner the specification fails to disclose a protocol and the proper parameters/controls when employing these experiments. The Examiner deems applicants'

previous response non-persuasive. Applicants argued that Example 10A describes such assay for measuring modulation of cyclic AMP levels.

In response to the Examiner, applicants submit that, contrary to the Examiner's assertions, in Example 10A of the specification reference was made to measuring cyclic AMP levels in determining receptor activity. Further, measuring cyclic AMP levels and changes in these levels is a well known method for determining agonist/antagonist activity on a receptor to those skilled in the art. See for example the Smith and Amer et al. publications which show that, prior to the filing date of the application, measuring cyclic AMP was a routine and well known assay in determining receptor activity. (Smith, C., The Cyclic AMP System and Drug Development, pp 187-203; and Amer, S., and McKinney, G., Possibilities for Drug Development Based on the Cyclic AMP System; Life Sciences (1973), Vol 13, pp 753-767; copies attached).

Therefore, Applicants submit claims 53-64 are enabled by the disclosure in the specification as under 35 USC §112, first paragraph. Accordingly, withdrawal of the rejection is respectfully requested.

The Examiner also maintained her rejection of claims 46, 53-64 under 35 USC §112, second paragraph, as being indefinite. The Examiner asserts that claims 53-64 are indefinite because of the recitation, "measuring a response indicative of the degree of agonist / antagonist activity". The Examiner asserts that it is unclear what response is being measured. Further, according to the Examiner the metes and bounds of these claims cannot be determined because the claims do not recite any assays and/or responses to discern agonist and/or antagonist activity. Similarly, according to the Examiner, in claims 55 and 60 it remains unclear whether modulation of cellular cyclic AMP levels measure an increase or decrease, which claims the Examiner

asserts are also unclear because they recite improper Markush groups. Further, claim 46 is objected to because the term "Il-8" should read "IL-8". Applicants have corrected the typographical error in claim 46, as amended.

In response to the Examiner applicants submit that measuring the compounds listed in the claims, which compounds are downstream from the receptor in a signal transduction pathway, is a well accepted method for determining agonist and/or antagonist activity. The assays and protocols for each particular compound to be measured are known and accepted by those skilled in the art.

Therefore, applicants submit that claims 53-64 clearly define the subject matter of the invention as under 35 USC §112, second paragraph. Accordingly, withdrawal of the rejection is respectfully requested. Further, the objection to claim 46 is moot in view of the correction of the typographical error, and withdrawal of the objection is respectfully requested.

Applicants submit that the present application is now in condition for allowance.

Reconsideration and favorable action are earnestly requested.

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[1] Measurement of Receptor-Stimulated Guanosine 5′-*O*-(γ-Thio)triphosphate Binding by G Proteins

By THOMAS WIELAND and KARL H. JAKOBS

Introduction

Many transmembrane signaling processes caused by extracellular hormones and neurotransmitters are mediated by receptors interacting with heterotrimeric ($\alpha\beta\gamma$) guanine nucleotide-binding proteins (G proteins) attached to the inner face of the plasma membrane. Agonist-liganded receptors apparently initiate activation of G proteins by catalyzing the exchange of guanosine 5′-diphosphate (GDP) by guanosine 5′-triphosphate (GTP) bound to the α subunits.[1,2] In membrane preparations and reconstituted systems, this activation process is frequently monitored by studying agonist stimulation of high-affinity GTPase, an enzymatic activity of G-protein α subunits.[3] However, the measurement of G-protein GTPase activity reflects steady-state kinetics of the overall G-protein activity cycle and not only the first step in the signal transduction cascade (i.e., the GDP/GTP exchange reaction). Furthermore, with regard to the molecular stoichiometry of receptor–G-protein interactions, only qualitative but not quantitative data can be obtained.

To study the initial steps of G-protein activation by agonist-liganded receptors in a quantitative manner, the binding of radiolabeled GTP analogs, which are not hydrolyzed by the GTPase activity of G-protein α subunits, to G proteins is determined. Of these GTP analogs, guanosine 5′-*O*-(γ-[^{35}S]thio)triphosphate ([^{35}S]GTPγS) is most frequently used. This nucleotide has a high affinity for all types of G proteins and is available with a relatively high specific radioactivity (1000–1400 Ci/mmol; physical half-life 87.4 days). Here we describe the measurement of receptor-induced binding of [^{35}S]GTPγS to membranous and detergent-solubilized G proteins and how this method can be adapted to different G proteins for an optimal response to receptor stimulation.

Materials

The [^{35}S]GTPγS (1000–1400 Ci/mmol) is obtained from Du Pont New England Nuclear (Bad Homburg, Germany). The substance is delivered

[1] A. G. Gilman, *Annu. Rev. Biochem.* **56**, 615 (1987).
[2] L. Birnbaumer, J. Abramovitz, and A. M. Brown, *Biochim. Biophys. Acta* **1031**, 163 (1990).
[3] D. Cassel and Z. Selinger, *Biochim. Biophys. Acta* **452**, 538 (1976).

in a buffer containing 10 m*M* *N*-tris(hydroxymethyl)methylglycine–NaOH, pH 7.6, and 10 m*M* dithiothreitol (DTT). To minimize decomposition, the solution is diluted 100-fold in this buffer and stored in aliquots at or below −70° before use. If the reagent is not stored at these recommended conditions, and after repeated freezing and thawing, chemical decomposition is rather high.

Unlabeled nucleotides and 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate (CHAPS) are from Boehringer Mannheim (Mannheim, Germany). *N*-Ethylmaleimide, *N*-formylmethionylleucylphenylalanine (fMet-Leu-Phe), isoproterenol, and carbachol are from Sigma (St. Louis, MO). Glass fiber filters (GF/C) are from Whatman (Clifton, NJ), and nitrocellulose filters (pore size 0.45 μm) are from Schleicher and Schuell (Keene, NH).

Membranes of various cells and tissues are prepared as previously described[4–7] and stored in aliquots at −70°. Before use in the binding assay, the membranes are thawed, diluted with 10 m*M* triethanolamine hydrochloride, pH 7.4, containing 5 m*M* EDTA, centrifuged for 10–30 min at 30,000 *g*, and resuspended in 10 m*M* triethanolamine hydrochloride, pH 7.4, at the appropriate membrane protein concentration.

Equipment

Incubator or water bath
Filtration funnel with vacuum pump
Cooled centrifuge (4°, up to 30,000 *g*) for membrane preparation
Ultracentrifuge with fixed-angle and swing-out rotors for preparation of membranes, solubilized proteins, and sucrose density gradient centrifugation
Shaker to equilibrate the filters with the scintillation cocktail
Liquid scintillation spectrometer
Freezer (preferably −70° or lower) for storage of membranes and [^{35}S]GTPγS

Measurement of Agonist-Induced [^{35}S]GTPγS Binding to G Proteins in Membranes

The assay is performed in 3-ml plastic reaction tubes. The assay volume is 100 μl. The final concentrations of the reaction mixture constituents

[4] P. Gierschik, M. Steisslinger, D. Sidiropoulos, E. Herrmann, and K. H. Jakobs, *Eur. J. Biochem.* **283**, 97 (1989).
[5] G. Hilf and K. H. Jakobs, *Eur. J. Pharmacol.* **172**, 155 (1989).
[6] D. S. Papermaster and W. J. Dreyer, *Biochemistry* **13**, 2438 (1974).
[7] G. Puchwein, T. Pfeuffer, and E. J. M. Helmreich, *J. Biol. Chem.* **249**, 3232 (1974).

are as follows: triethanolamine hydrochloride (pH 7.4), 50 m*M*; $MgCl_2$, 5 m*M*; EDTA, 1 m*M*; DTT, 1 m*M*; NaCl, 0–150 m*M*; GDP, 0–100 μM; [^{35}S]GTPγS, 0.3–0.5 n*M* (~50 nCi). The incubation temperature and membrane concentration as well as the concentrations of NaCl and GDP have to be adjusted to the individual cell type and the G-protein subtype activated by the receptor under study.

1. The reaction mixture (40 μl) together with the receptor agonist or its diluent (10 μl) are thermally preequilibrated for 5 min at the desired reaction temperature.
2. The binding reaction is started by addition of the membrane suspension (50 μl) and vortexing.
3. Samples are incubated for the appropriate incubation time, for example, 60 min at 30°.
4. The incubation is terminated by the addition of 2.5 ml of an ice-cold washing buffer (50 m*M* Tris-HCl, pH 7.5, 5 m*M* $MgCl_2$).
5. This mixture is passed through the filtration funnel. For systems containing only membrane-bound G proteins, Whatman GF/C glass fiber filters are used. In systems containing soluble G proteins (e.g., transducin), nitrocellulose filters are required.
6. The reaction tube is washed two times with 2.5 ml of the washing buffer, and this solution is also passed through the same filter.
7. The filter is additionally washed two times with 2.5 ml of the washing buffer and then dried at room temperature.
8. The dried filters are put into 5-ml counting vials and equilibrated with 4 ml of a scintillation cocktail for 20 min at room temperature by moderate shaking. Any commercially available scintillation cocktail suitable for counting of ^{35}S can be used. Also a self-made cocktail consisting of 2 liters toluene, 1 liter Triton X-100, 15 g 2,5-diphenyloxazole, and 3 g 2,2′-*p*-phenylenebis(4-methyl-5-phenyloxazole) can be used.

Application to Various Cell Types and Different G Proteins

In membranes of various cell types, including human neutrophils,[8] human platelets,[9] human leukemia cells (HL-60),[10,11] rat myometrium,[12]

[8] R. Kupper, B. Dewald, K. H. Jakobs, M. Baggiolini, and P. Gierschik, *Biochem. J.* **282**, 429 (1992).
[9] C. Gachet, J.-P. Cazenave, P. Ohlmann, G. Hilf, T. Wieland, and K. H. Jakobs, *Eur. J. Biochem.* **207**, 259 (1992).
[10] P. Gierschik, R. Moghtader, C. Straub, K. Dieterich, and K. H. Jakobs, *Eur. J. Biochem.* **197**, 725 (1991).
[11] T. M. Schepers, M. E. Brier, and K. R. McLeish, *J. Biol. Chem.* **267**, 159 (1992).
[12] C. Liebmann, M. Schnittler, M. Nawrath, and K. H. Jakobs, *Eur. J. Pharmacol.* **207**, 67 (1991).

and pig atrium,[13] as well as in reconstituted systems,[14-16] an agonist-induced increase in [^{35}S]GTPγS binding to G proteins is optimal at millimolar concentrations (1–5 m*M*) of free Mg^{2+}. In general, the membrane concentration (and the incubation time) should be kept at a level at which not more than 20–30% of the total added [^{35}S]GTPγS is bound. For most tissues, 1–10 μg of membrane protein/tube is appropriate. Even lower amounts are required in systems with a high content of G proteins (e.g., rod outer segment membranes). To optimize the response to agonist-activated receptors, the assay system has to be adapted to the respective G protein interacting with these receptors. The major strategy is to minimize the agonist-independent binding to G proteins without lowering the agonist-induced binding.

Regulation by Guanosine 5'-Diphosphate, Sodium Chloride, and Temperature

As reported for native HL-60 plasma membranes, the G proteins are initially in a GDP-liganded form, and, thus, the agonist-independent binding of [^{35}S]GTPγS to G proteins is limited by the dissociation of GDP from the binding sites.[17] Two components may contribute to this agonist-independent reaction: (1) spontaneous, receptor-independent dissociation of G-protein-bound GDP and (2) GDP release induced by agonist-unliganded receptors.[18] The extent of the spontaneous agonist-independent release of GDP from G proteins differs between various cell types and apparently even more between different G-protein subtypes.

Therefore, several approaches can be used to adapt the assay to the respective system. In Fig. 1, receptor-stimulated [^{35}S]GTPγS binding to three different G proteins is shown. In HL-60 membranes, G proteins of the G_i subtype interacting with chemotactic receptors are predominantly seen.[17] When these membranes are incubated with [^{35}S]GTPγS at 30° (Fig. 1A), which condition leads to a rapid spontaneous dissociation of GDP from the G proteins, a rather high basal binding rate is obtained, and addition of a receptor agonist (e.g., fMet-Leu-Phe) does not cause a further increase in binding. GDP has to be added to keep the G_i proteins in the GDP-liganded form required for optimal receptor action. The additional presence of NaCl at an optimal concentration of 100–150 m*M* increases

[13] G. Hilf, P. Gierschik, and K. H. Jakobs, *Eur. J. Biochem.* **186**, 725 (1989).
[14] A. B. Fawzi and J. K. Northup, *Biochemistry* **29**, 3804 (1990).
[15] M. R. Tota, K. R. Kahler, and M. I. Schimerlik, *Biochemistry* **26**, 8175 (1987).
[16] V. A. Florio and P. C. Sternweis, *J. Biol. Chem.* **264**, 3909 (1989).
[17] T. Wieland, J. Kreiss, P. Gierschik, and K. H. Jakobs, *Eur. J. Biochem.* **205**, 1201 (1992).
[18] G. Hilf and K. H. Jakobs, *Eur. J. Pharmacol.* **225**, 245 (1992).

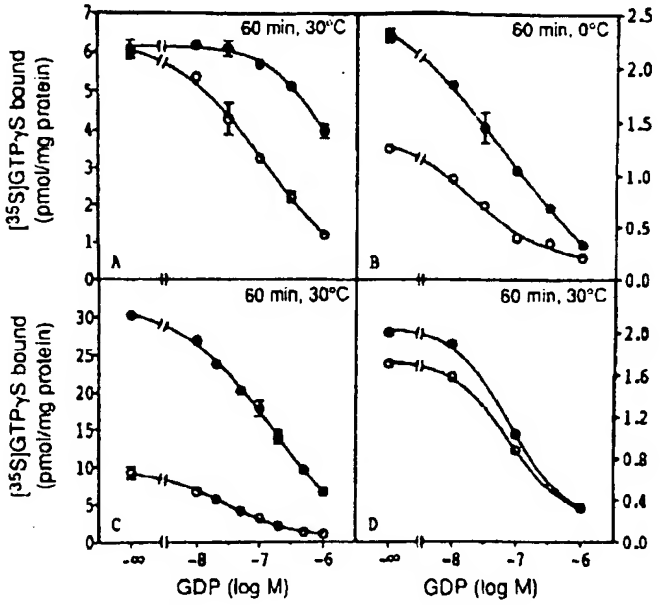

FIG. 1. Regulation by GDP of receptor-stimulated [^{35}S]GTPγS binding to different G proteins. HL-60 membranes (A, B), bovine rod outer segment membranes (C), and turkey erythrocyte membranes (D) were incubated with approximately 0.5 n*M* [^{35}S]GTPγS in a reaction mixture containing 50 m*M* triethanolamine hydrochloride, pH 7.4, 5 m*M* $MgCl_2$, 1 m*M* EDTA, 1 m*M* DTT, 150 m*M* NaCl, and GDP, at the indicated concentrations, for 60 min at either 30° (A, C, D) or 0° (B) in the absence (○) and presence of receptor stimuli (●) (10 μ*M* fMet-Leu-Phe in A and B, bright white light in C, 10 μ*M* isoproterenol in D). For further details, see text. Means plus or minus the standard deviation of triplicate determinations are given.

further the inhibitory influence of GDP on basal binding, probably by uncoupling G proteins from unoccupied receptors.[10,19] In membranes from other cell types, addition of KCl may give similar or even better results.

In contrast, when binding is performed at 0° with a low spontaneous rate of GDP release, basal binding of [^{35}S]GTPγS is much lower, and, most importantly, fMet-Leu-Phe-induced binding is observed without addition of GDP (Fig. 1B). At 0°, addition of either GDP or NaCl exerted only negative effects on receptor-induced binding.[17] Thus, for measuring receptor-stimulated binding of [^{35}S]GTPγS to G_i or G_o[16] proteins either incubation at low temperature (e.g., 0°) or, at higher temperatures

[19] P. Gierschik, D. Sidiropoulos, M. Steisslinger, and K. H. Jakobs, *Eur. J. Pharmacol.* **172**, 481 (1989).

(25°–37°), addition of GDP (0.1–10 μM) and NaCl (100–150 mM) is recommended.

The retinal G protein transducin is known to have a very low spontaneous dissociation rate for GDP, even when incubated at higher temperatures.[14] Thus, as shown in Fig. 1C, addition of GDP is not required for stimulation of GTPγS binding by light-activated rhodopsin (by illumination of bovine rod outer segment membranes adapted to dim red light). Because the binding of GTPγS observed in dim red light is more sensitive to inhibition by GDP and ionic strength than that observed in bright white light, addition of GDP and NaCl (or KCl) will result in a marked increase in the level (-fold) stimulation by illumination.

The membranous G_s protein seems to have a low spontaneous rate of GDP dissociation. As shown in Fig. 1D, agonist (isoproterenol) activation of β-adrenoceptors interacting with G_s proteins induces a slight but significant increase in binding of [^{35}S]GTPγS when turkey erythrocyte membranes are incubated for 60 min at 30°. Similarly, as described for HL-60 membranes at 0°, addition of GDP and NaCl only decreases receptor-induced binding and, therefore, should be avoided in this system. Moreover, in turkey erythrocyte membranes, the isoproterenol-independent binding of [^{35}S]GTPγS is probably due to binding to GDP-free G proteins other than G_s. Thus, additional strategies have to be used to reduce the agonist-independent binding of the radioligand.

Treatment of Membranes with N-Ethylmaleimide and/or Unlabeled GTPγS to Reduce Agonist-Independent Binding of [^{35}S]GTPγS

1. Turkey erythrocyte membranes (3–5 mg protein) are incubated for 30 min on ice in 50-ml centrifugation tubes in a reaction mixture (total volume 9 ml) containing 50 mM triethanolamine hydrochloride, pH 7.4, 10 mM *N*-ethylmaleimide (NEM), and 1 mM EDTA.

2. One milliliter of a 150 mM 2-mercaptoethanol solution is added to stop protein alkylation by NEM, followed by the addition of 30 ml of ice-cold 10 mM triethanolamine hydrochloride, pH 7.4.

3. Membranes are pelleted by centrifugation (10 min at 30,000 g), resuspended in 1 ml of 10 mM triethanolamine hydrochloride, pH 7.4, and further treated with unlabeled GTPγS or stored in aliquots at −70°.

4. The NEM-treated or untreated membranes are incubated for 30 min at 30° in a reaction mixture (final volume 2 ml) containing 50 mM triethanolamine hydrochloride, pH 7.4, 5 mM $MgCl_2$, 1 mM EDTA, and 1 μM unlabeled GTPγS.

5. After addition of 8 ml of ice-cold 10 mM triethanolamine hydrochloride, pH 7.4, membranes are pelleted by centrifugation (10 min at

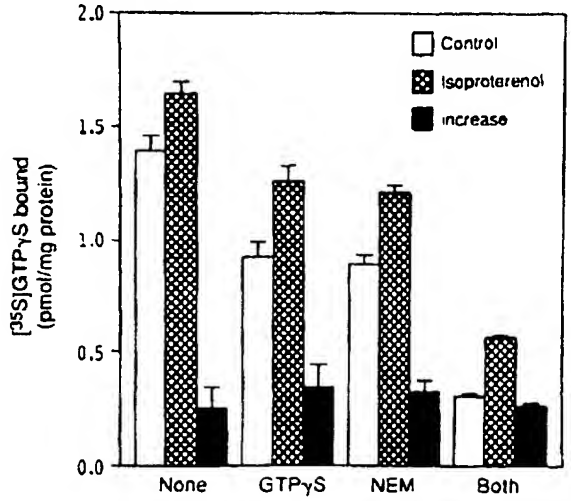

FIG. 2. Optimization of β-adrenoceptor-induced [^{35}S]GTPγS binding to turkey erythrocyte membrane G_s proteins. Turkey erythrocyte membranes were preincubated with either 10 m*M* NEM, 1 μ*M* unlabeled GTPγS, or both agents as described in the text. Thereafter, binding of 0.45 n*M* [^{35}S]GTPγS to untreated (none), GTPγS-treated, NEM-treated, and membranes treated with both agents (both) (10 μg protein each) was determined in the absence (open bars) and presence of 10 μ*M* isoproterenol (cross-hatched bars). The β-adrenoceptor-induced increase in [^{35}S]GTPγS binding is given by the filled bars. Means of assay triplicates plus or minus the standard deviation are shown.

30,000 *g*), resuspended in 1 ml of 10 m*M* triethanolamine hydrochloride, pH 7.4, and stored in aliquots at −70°.

The thiol group-alkylating agent NEM has been shown to modify cysteine residues on pertussis toxin-sensitive G proteins. Similar to $G_{i/o}$ proteins ADP-ribosylated by pertussis toxin, NEM-treated $G_{i/o}$ proteins cannot be activated by receptors.[20] When turkey erythrocyte membranes are preincubated with NEM, the isoproterenol-independent binding of GTPγS is lowered by about 35%, whereas the β-adrenergic agonist-induced binding is not altered (Fig. 2). A similar reduction in agonist-independent binding is obtained by treatment of the membranes with a saturating concentration of unlabeled GTPγS in the absence of agonists. Most likely, unlabeled GTPγS binds preferentially to G proteins with a high spontaneous GDP release and, therefore, prevents binding of radiolabeled GTPγS to these G proteins. In turkey erythrocyte membranes, a combination of

[20] M. Ui, *in* "ADP-Ribosylating Toxins and G Proteins" (J. Moss and M. Vaughan, eds.), p. 45. American Society for Microbiology, Washington, D.C., 1990.

both treatments gives the best results in lowering isoproterenol-independent binding (~75% reduction) without affecting the β-adrenoceptor-mediated increase in [^{35}S]GTPγS binding. In human platelet membranes (not shown), NEM treatment alone was sufficient for improving relative prostaglandin E_1-induced binding of [^{35}S]GTPγS to G_i. Thus, to study agonist-induced GTPγS binding to G_s in membranes of other tissues, a stepwise treatment and testing after each treatment should be performed.

Stoichiometry of Receptor–G-Protein Interactions

For quantitative analysis of the stoichiometry of receptor–G-protein interactions, the amount of receptors under study as well as the amount of G proteins present in the given membrane preparation have to be measured. The concentration of receptors is determined by binding saturation analysis with receptor-specific radioligands. The concentration of G proteins activated by the receptors is analyzed by measuring agonist-stimulated binding of [^{35}S]GTPγS under conditions optimal for the given receptor and G protein under study (see above). Furthermore, the incubation time has to be adjusted to give a maximal absolute increase in agonist stimulation of [^{35}S]GTPγS binding. In addition, the assay contains increasing concentrations of GTPγS (up to 0.5–1 μ*M*) in the absence and presence of the receptor agonist at a maximally effective concentration. Under ideal conditions, the resulting binding data can be analyzed by Scatchard transformation. Such an analysis performed for formyl peptide receptor-stimulated binding of GTPγS to G proteins in HL-60 membranes is exemplified in Fig. 3. In these membranes, a stoichiometry of up to 20 G proteins activated by a one agonist-liganded formyl peptide receptor has been determined by this technique.[10]

Measurement of Agonist-Induced [^{35}S]GTPγS Binding in Solubilized Systems

Solubilization of Membrane Proteins and Sucrose Density Gradient Centrifugation

1. Membrane aliquots are thawed, pelleted by centrifugation (30 min at 30,000 *g*) and resuspended in solubilization buffer [Tris-HCl, pH 7.4, 50 m*M*; EDTA, 1 m*M*; DTT, 1 m*M*; CHAPS, 1% (by mass)] at a protein concentration of 1% (by mass).
2. Proteins are solubilized by mild agitation for 1 hr at 4°.
3. The mixture is centrifuged for 1 hr at 200,000 *g*, and the supernatant is passed through a nitrocellulose membrane (pore size 0.45 μm).

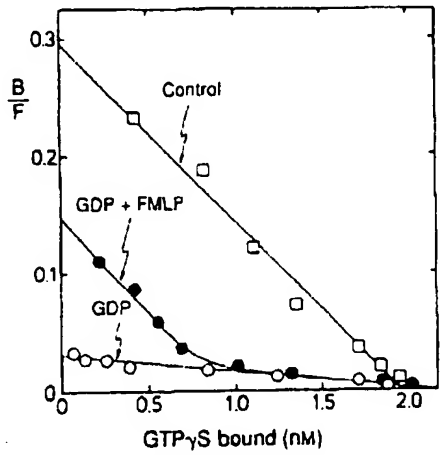

FIG. 3. Quantification of G proteins activated by an agonist-activated receptor in HL-60 membranes. Binding of GTPγS to HL-60 membranes (3 μg protein) was determined in a total volume of 100 μl in the absence (control, □) and presence of 1 μ*M* GDP (○) or 1 μ*M* GDP plus 10 μ*M* fMet-Leu-Phe (GDP + FMLP, ●) at increasing concentrations of GTPγS (0.3–500 n*M*). A Scatchard analysis of the binding data is shown. For further experimental details, see text.

4. Beckman 5-ml Ultraclear tubes are stepwise filled by sublayering with 1.2 ml of each gradient buffer containing 50 m*M* Tris-HCl, pH 7.4, 1 m*M* EDTA, 1 m*M* DTT, 0.3% CHAPS, and 5, 10, 15, or 20% sucrose. Gradients are kept for 3 hr at room temperature for linearizing.

5. Next, 150 μl solubilizate and 50 μl solubilization buffer including molecular weight marker proteins (e.g., catalase and cytochrome *c*) are layered on each gradient.

6. Gradients are centrifuged for 12 hr at 50,000 rpm in a Beckman SW 50 rotor. Thereafter, tubes are punctured and fractions (~250 μl) are collected by trapping drops into 1.5-ml Eppendorf plastic tubes.

7. The distribution of the marker proteins is measured by spectrometric methods.

Receptor-Stimulated [^{35}S]GTPγS Binding in Fractions Containing Both Receptor and G Proteins

The distribution of the receptor in the sucrose density gradient fractions is assessed by measuring binding of radiolabeled agonists or antagonists for the respective receptor. For identification of G proteins, 10 μl of each fraction is incubated for 60 min at 30° in 3-ml plastic reaction tubes with 40 μl of a reaction mixture containing 25 m*M* HEPES, pH 8.0, 20 m*M*

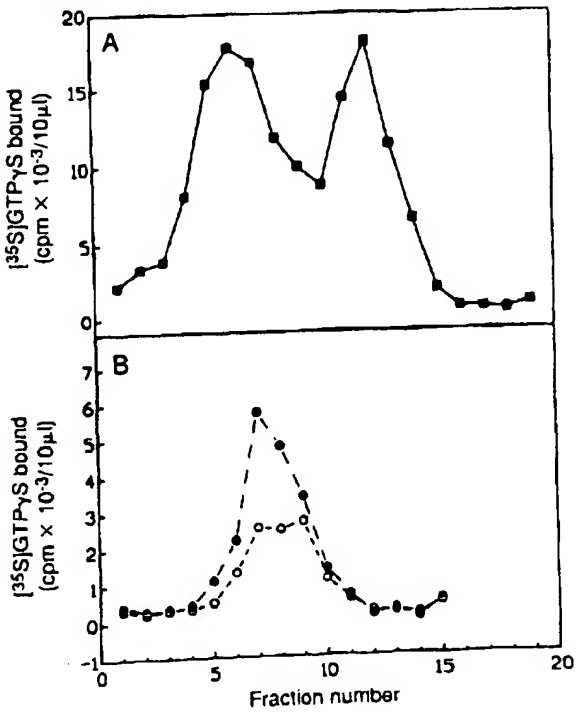

FIG. 4. Receptor-stimulated [^{35}S]GTPγS binding in sucrose density gradient fractions of solubilized pig atrial membranes. Pig atrial membranes were solubilized and sucrose gradient fractions prepared as described in the text. (A) The distribution of G proteins was determined by measuring binding of 10 n*M* [^{35}S]GTPγS in 10-μl aliquots of each fraction. (B) Muscarinic acetylcholine receptor-stimulated binding of [^{35}S]GTPγS (0.6 n*M*) was determined in 10-μl aliquots of the fractions in the absence (○) and presence of 100 μ*M* carbachol (●). For experimental details of [^{35}S]GTPγS binding, see text.

$MgCl_2$, 1 m*M* EDTA, 1 m*M* DTT, 100 m*M* NaCl, 0.124% Lubrol PX, and 10 n*M* GTPγS, including 0.6–0.8 n*M* [^{35}S]GTPγS. The incubation is terminated by the addition of 2.5 ml of ice-cold washing buffer (50 m*M* Tris-HCl, pH 7.5, 5 m*M* $MgCl_2$). The mixture is rapidly passed through nitrocellulose filters, and filters are washed and treated as described above. As shown for solubilized G proteins from pig atrial membranes,[21] two distinct peaks of G proteins are obtained by sucrose density gradient centrifugation (Fig. 4A). The first peak with a higher sedimentation coefficient (about 6.4 S) contains essentially heterotrimeric G proteins, whereas

[21] G. Hilf and K. H. Jakobs, *Cellular Signalling* 4, 787 (1992).

the second peak (sedimentation coefficient about 3.3 S) is mainly due to small molecular mass G proteins.

In fractions containing both heterotrimeric G proteins and receptor(s), agonist-induced binding of [^{35}S]GTPγS can be measured using assay conditions similarly as described for membrane preparations. For example, 10 μl of each sucrose density gradient fraction obtained from solubilized pig atrial membranes are incubated in a total volume of 60 μl for 90 min at 25° with a reaction mixture containing 50 m*M* Tris-HCl, pH 7.4, 5 m*M* $MgCl_2$, 1 m*M* EDTA, 1 m*M* DTT, 100 m*M* NaCl, 5 μ*M* GDP, and 0.6 n*M* [^{35}S]GTPγS, with or without the muscarinic agonist carbachol at 100 μ*M*. The final concentration of the detergent CHAPS should be maintained at 0.1%. At lower concentrations, precipitation of solubilized proteins may occur, whereas higher CHAPS concentrations will lower the agonist-induced binding. The reaction is stopped, and binding of [^{35}S]GTPγS to solubilized G proteins is analyzed by filtration through nitrocellulose filters as described above. In fractions containing both muscarinic acetylcholine receptors and heterotrimeric G proteins, carbachol stimulates binding of [^{35}S]GTPγS to solubilized G proteins by about 2-fold (Fig. 4B). Similar data have been obtained for fMet-Leu-Phe-stimulated binding of [^{35}S]GTPγS in sucrose density gradient fractions of HL-60 membranes containing both solubilized formyl peptide receptors and G_i proteins (not shown). These fractions may then be pooled and used for identification and/or purification of the G protein(s) interacting with the respective receptors.

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[2] Receptor-Stimulated Hydrolysis of Guanosine 5'-Triphosphate in Membrane Preparations

By PETER GIERSCHIK, THOMAS BOUILLON, and KARL H. JAKOBS

Introduction

The most prominent functional characteristic of signal-transducing heterotrimeric guanine nucleotide-binding proteins (G proteins) is their cyclic movement through a series of activation and deactivation steps, which is characterized by changes in the nature of the bound guanine nucleotide and in the status of the subunit association. Activation of G proteins is initiated by the release of guanosine 5'-diphosphate (GDP) from the heterotrimeric G protein, followed by the binding of guanosine 5'-triphosphate (GTP) and the dissociation of the αβγ heterotrimer into GTP-liganded α subunit and free βγ dimer. Both free α subunits and free βγ

8

Measurement of Agonist-Stimulated [^{35}S]GTPγS Binding to Cell Membranes

Sebastian Lazareno

1. Introduction

This chapter describes a functional assay that measures the increase in guanine nucleotide exchange at G proteins in cell membranes, resulting from agonist binding to G protein-coupled receptors (GPCRs), by monitoring the binding of a radiolabeled, hydrolysis-resistant analog of GTP, [^{35}S]GTPγS, in the presence of unlabeled GDP.

The function of GPCR activation is to stimulate GTP/GDP exchange at G proteins *(1)* (Fig. 1). In a cell, the guanine nucleotide exchange cycle is initiated by the binding of an agonist-occupied (or "activated") GPCR to a heterotrimeric G protein in the cell membrane. This stimulates the dissociation of GDP from the α-subunit of the G protein, allowing endogenous GTP to bind in its place. This in turn causes the dissociation of the receptor and the Gα-GTP and Gβγ-subunits of the G protein. The Gα-GTP and Gβγ-subunits can each activate effectors, such as adenylyl cyclase, PLC, and ion channels *(1)*. The Gα-GTP is inactivated by an intrinsic GTPase, which hydrolyzes the GTP to GDP; Gα-GDP in turn inactivates the Gβγ by binding to it, resulting in an inactive GDP-containing heterotrimeric G protein ready for the next activation cycle.

This process can be monitored in vitro by incubating cell membranes containing G proteins and GPCRs with GDP and [^{35}S]GTPγS. Binding of [^{35}S]GTPγS, like GTP, stimulates dissociation of Gα-[^{35}S]GTPγS and Gβγ, but, unlike GTP, [^{35}S]GTPγS is relatively resistant to hydrolysis by the intrinsic GTPase of Gα, dissociates slowly from Gα, and therefore accumulates in the membranes. The effect of receptor activation on [^{35}S]GTPγS binding in the presence of GDP is catalytic, i.e., it increases the rate of [^{35}S]GTPγS binding

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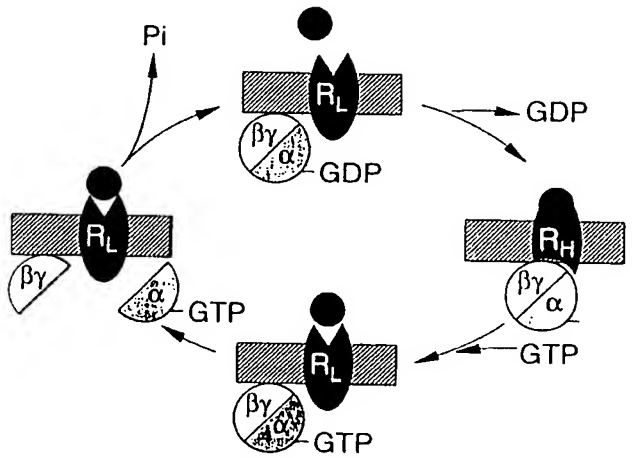

Fig. 1. Guanine nucleotide (GDP/GTP) exchange cycle at G proteins (αbγ) stimulated by agonist (A) binding to receptor (high agonist-affinity state, R_H, or low agonist-affinity state, R_L).

rather than the equilibrium level of [^{35}S]GTPγS binding. So this is intrinsically a nonequilibrium binding assay.

The assay of agonist-stimulated [^{35}S]GTPγS binding has a number of useful features:

1. It can use the same membrane preparations and assay conditions used in studies that measure radioligand binding to the GPCR, thus allowing a direct comparison of agonist action and agonist occupancy of the receptor;
2. It allows a measure of agonist action in systems in which the subsequent effector mechanisms are unknown;
3. It allows a direct comparison of receptor activation with receptors that activate different G protein regulated effector systems; and
4. It is convenient, easy, quick, and relatively accurate.

The method used to measure agonist-stimulated [^{35}S]GTPγS binding is a modification of methods described by Jakobs and colleagues *(2,3)*.

2. Materials

1. [^{35}S]GTPγS (1000–1400 Ci/mmol) (*see* Note 1).
2. Cell membranes: Prepare as described in Section 3.1.
3. Homogenizing buffer: 20 m*M* HEPES/Na HEPES, pH 7.4, 10 m*M* EDTA. Protease inhibitors and reducing agents may also be included if required (*see* e.g., Sweeney *(4)*). It is convenient to prepare or purchase stock solutions of 500 m*M* EDTA, which is stored at room temperature, and 1*M* HEPES/Na HEPES, which is stored at 4°C. A mixture of equal volumes of 1*M* HEPES and 1*M* Na HEPES should have a pH of about 7.5 when diluted to 20 m*M*.
4. Membrane storage buffer: 20 m*M* HEPES/Na HEPES, pH 7.4, 0.1 m*M* EDTA.
5. Assay buffer: 20 m*M* HEPES/Na HEPES, pH 7.4, 100 m*M* NaCl, 10 m*M* $MgCl_2$, 1 μ*M* GDP (*see* Note 2).
6. Washing buffer: 10 m*M* sodium phosphate buffer, pH 7.4 (*see* Note 5). A mixture of Na_2HPO_4 and NaH_2PO_4, each at 10 m*M*, in the ratio 1.4:0.6 v:v, has a pH of about 7.4.

3. Methods

3.1. Preparation of Membranes

1. Wash the cells twice with 10 mL ice-cold homogenization buffer and scrape them off the plate with a teflon spatula and 2X 3 mL ice-cold homogenization buffer.
2. Homogenize the cells with three or four 5-s bursts of a Polytron homogenizer (setting 6, with 30 s on ice between bursts).
3. Dilute the homogenate to 30 mL with ice-cold homogenization buffer and centrifuge at 40,000*g* for 10 min at 4°C.
4. Discard the supernatant and rehomogenize the pellet in 30 mL ice-cold membrane storage buffer. Centrifuge at 40,000*g* for 10 min at 4°C.
5. Repeat step 4.
6. Resuspend the pellet in about 5 mL ice-cold membrane storage buffer.
7. Determine the protein content (*see* Note 3).
8. Dilute the membrane preparation to 2 mg/mL protein with ice-cold membrane storage buffer and store in 0.5 mL aliquots at –70°C.

3.2. [^{35}S]GTPγS Binding Assay: Measurement of Agonist Effect

1. Thaw an aliquot of frozen membranes and dilute to a concentration of 20 μg protein/mL in ice-cold assay buffer containing 1 μ*M* GDP (*see* Note 2). Store on ice.
2. Prepare 5-mL polystyrene test tubes in triplicate (*see* Note 4) with 10 μL of each Agonist test agent made up to 100 times the final concentration in the assay.
3. Thaw an aliquot of [^{35}S]GTPγS and dilute a portion to a concentration of about 10 n*M* in assay buffer; if the original [^{35}S]GTPγS has been stored at 10-fold dilution, then dilute it a further 100-fold (*see* Note 1).
4. Add the diluted [^{35}S]GTPγS to the diluted membranes, 10 μL label for each mL of membranes, and mix well. At 4°C, in the presence of GDP, binding of 0.1 n*M* [^{35}S]GTPγS is very slow.
5. Distribute 1-mL aliquots of membranes + GDP + [^{35}S]GTPγS to the prepared tubes.
6. Incubate the samples at 30°C for 30 min.
7. Filter the samples over wetted (with water) glass fiber filters (Whatman GF/B) and wash twice with 3 mL ice-cold washing buffer (*see* Notes 5, 6, and 13).

8. Distribute the filter disks to scintillation vials and add scintillation fluid (*see* Note 7).
9. Distribute 10 µL diluted [^{35}S]GTPγS (or 100 µL membranes + label) in duplicate to scintillation vials for measurement of added label.
10. Determine the radioactivity in each sample using scintillation spectroscopy (*see* Note 7).

3.3. [^{35}S]GTPγS Binding Assay: Measurement of Antagonist Affinity

Potent antagonists often have slow dissociation kinetics, so expose the membranes to the agonist and antagonist for sufficient time, e.g., 30 min, for binding equilibrium of both ligands to be attained before addition of [^{35}S]GTPγS to the assay.

1. *See* Note 8 for experimental design.
2. Perform steps 1–3 of Section 3.2.
3. Distribute 1-mL membranes + GDP to the prepared tubes.
4. Incubate the samples at 30°C for 30 min.
5. Add 10 µL [^{35}S]GTPγS to the samples.
6. Perform steps 6–10 of Section 3.2.
7. *See* Note 9 for data analysis.

3.4. [^{35}S]GTPγS Binding Assay: Quantitation of Allosteric Modulator

Some GPCRs, such as the muscarinic and adenosine A_1 receptors, contain a second site at which agents can bind, with the effect of modulating the affinity of a directly acting ligand (such as the endogenous agonist) *(5)*.

1. Use a Schild analysis design (*see* Note 8).
2. If the allosteric agent has rapid dissociation kinetics (i.e., a $K_d > 10^{-7}M$), then use the procedure of Section 3.2.; otherwise, use the procedure of Section 3.3.
3. *See* Note 10 for data analysis.

3.5. [^{35}S]GTPγS/[^{3}H]Antagonist Dual Label Binding Assay: Simultaneous Measurement of Agonist Binding and Functional Effect (6)

Figure 2 shows an example.

1. Prepare tubes with 10 µL agonist and 1-mL membranes containing GDP.
2. Add 10 µL of [^{3}H]antagonist radioligand and incubate at 30°C until binding is at equilibrium.
3. Add 10 µL diluted [^{35}S]GTPγS to each tube and continue the incubation for 30 min.
4. Filter over wetted (with water) filters and wash twice with 3 mL ice-cold washing buffer (*see* Notes 5 and 13).

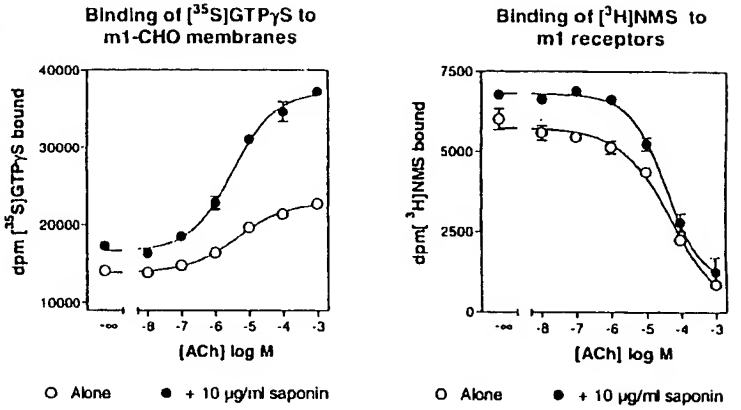

Fig. 2. Dual-label experiment to measure simultaneously the effect of saponin on ACh stimulation of [^{35}S]GTPγS binding to G proteins and inhibition of antagonist radioligand (^{3}H-NMS) binding to muscarinic m1 receptors in CHO cell membranes. 0.1 µ*M* GDP was present. The assay was conducted in duplicate.

5. Distribute the filter disks to scintillation vials and add scintillation fluid (*see* Note 11).
6. Determine the radioactivity in each filter using the dual ^{3}H/^{35}S label program provided with the scintillation counter (*see* Notes 11 and 12).
7. *See* Note 13 for problems of contamination.

3.6. [^{35}S]GTPγS Binding Assay: Improving the Signal-to-Noise Ratio

1. Optimize the assay as described in Notes 2 and 5. Check incubation time and temperature.
2. Purify the plasma membrane preparation with sucrose density gradient centrifugation (e.g., ref. 7).
3. Include 10 µg/mL saponin in the assay (~1:1 saponin:protein). We have found (ref. *8* and S. Lazareno, unpublished observations; *see* Fig. 2) that this increases the signal-to-noise ratio without perturbing the pharmacological characteristics of the preparation, though possible effects of saponin on the pharmacological parameters being studied should be assessed. Other authors have used alamethicin as a permeabilizing agent *(9)*.
4. Stop the reaction by adding high concentrations of unlabeled GTPγS (e.g., 10 µ*M*) and competitive antagonist. Allow the label to dissociate for 45 min before filtration. This may yield a modest (10–30%) increase in the signal-to-noise ratio.

4. Notes

1. [^{35}S]GTPγS is currently supplied by NEN (New England Nuclear, Du Pont, UK) at a concentration of about 10^{-5} *M* in a buffer containing 10 m*M* tricine and 10 m*M* dithiothreitol, pH 7.6. It should be diluted at least 10-fold in this buffer and stored at −70°C in aliquots sufficient for two or three assays, in order to minimize radiochemical decomposition. According to the volumes and dilutions recommended in this chapter, this will result in a concentration of 1 μ*M* dithiothreitol in the assay.
2. The precise concentrations of NaCl and $MgCl_2$, and particularly of GDP, required for the optimal signal-to-noise ratio (maximal agonist-stimulated binding/basal binding) should be determined for each receptor-G protein preparation. For some receptors, e.g., muscarinic m1 receptors expressed in CHO cells, agonists stimulate [^{35}S]GTPγS binding in the absence of GDP, and inclusion of up to 0.1 μ*M* GDP in the [^{35}S]GTPγS binding assay reduces basal binding without reducing stimulated binding. For other receptors, such as the muscarinic m2 receptor, GDP is absolutely required for agonist stimulation, and 1 μ*M* GDP was found to provide optimal results; 10 μ*M* GDP was required with the adenosine A_1 receptor *(8,10)*. In all cases, sufficient bound counts must be obtained for accurate measurement, but not more than about 10% of added [^{35}S]GTPγS should be bound, so less m1 receptor membrane is used (5 μg protein/mL) than m2 receptor membrane (20 μg/mL). Note that GDP reduces agonist potency in the [^{35}S]GTPγS binding assay *(6)*.
3. The concentrated membranes may be stored at −70°C and later thawed for protein determination, dilution, and refreezing, without loss of stimulated [^{35}S]GTPγS binding activity.
4. Because this is a binding assay, the variability between replicates is usually small, 2–5% of the mean counts, so duplicate measures are often sufficient. On the other hand, the stimulation of binding by agonist above basal values may be less than twofold, so triplicate or even quadruplicate determinations may be necessary.
5. We find cold water to be acceptable for washing the filters. The filter blank, measured in the absence of membranes, is usually less than 0.5% of added label. Occasionally, however, much larger filter blanks occur, for reasons which are as yet unresolved. Large filter blanks may be reduced by washing in phosphate buffer. Nonspecific binding, measured in the presence of 10 μ*M* unlabeled GTPγS, is usually close to filter blank levels.
6. As far as possible, experiments should be designed so that all the data contributing to an experimental question are obtained from a single filter. If this is not possible, then two or more filters should be used, each containing a single replicate of each experimental condition. In this way the variability between filters caused by small differences in washing procedure or incubation time is evenly distributed across all experimental conditions. In order to minimize possible position effects of the cell harvester, the second replicates may be filtered in reverse order. This is easily achieved by reversing the order in which samples are filtered, and then reversing the filter before distributing the filter disks to scintillation vials.
7. Filters dried before addition of scintillation fluid can be counted immediately. Counting wet filters will provide a good indication of the result of the assay, but for accurate data the prepared vials should be left overnight and then inverted and shaken repeatedly to ensure that the water in the filter has completely dissolved in the scintillation fluid.
8. The affinity of a competitive antagonist is estimated in functional studies by constructing agonist concentration–effect curves, alone and in the presence of various fixed concentrations of antagonist, and analyzing the data with Schild analysis *(11)*. This design allows the detection of any changes in basal activity, the E_{max}, or the shape of the agonist curve in the presence of antagonist. If it can be assumed that the antagonist does not alter E_{max} or agonist slope, it is simpler and more efficient to estimate antagonist affinity with this assay using an inhibition curve design. In this case, a minimum of two concentration–effect curves is required: an agonist curve alone, and an antagonist curve in the presence of a fixed agonist concentration. The effect of the antagonist on basal activity should also be measured, though this information may not contribute to the data analysis.
9. Although the data may be analyzed using linear transformations and even *"by eye"* interpolations from graphs *(12,13)*, ideally the data should be analyzed using nonlinear regression analysis. The manual provided with the Prism program (GraphPad) contains an introduction to nonlinear regression and other useful topics: the relevant chapters are freely available on the World Wide Web (http://www.graphpad.com). Some programs (e.g., Prism, *see* ref. *14*) can only handle one independent variable, e.g., drug concentration. Others (e.g., SigmaPlot [Jandel] and modern spreadsheets *[15]*) can handle two or more independent variables, e.g., agonist and antagonist concentrations. Equations containing one independent variable are marked (*); those containing two independent variables are marked (**).

   Schild plot designs can be analyzed in the traditional way *(11)* or by fitting the complete data set, together with agonist [A] and antagonist [B] concentrations, directly to the integrated logistic-Schild equation *(12,13)*:

$$\text{Effect} = \frac{E_{max} - \text{basal}}{1 + \left\{\frac{[EC_{50}]}{[A]} \cdot \left[\left(\frac{[B]}{K_b}\right)^s + 1\right]\right\}^b} + \text{basal} \qquad (1)\ (**)$$

   to yield estimates of basal activity, E_{max}, agonist EC_{50}, agonist slope factor *b*, antagonist dissociation constant K_b, and Schild slope *s*.

   Inhibition curve designs can be analyzed in three ways *(12,13)*.

   a. Using a SigmaPlot-type program, fit the complete data set to Eq. (1).
   b. Using a Prism-type program:
      i. Fit the agonist curve to a logistic function; Eq. (2).

$$\text{Effect} = \frac{E_{max} - \text{basal}}{1 + \left\{\frac{[EC_{50}]}{[A]}\right\}^b} + \text{basal} \qquad (2)\ (*)$$

ii. Fit the antagonist curve to Eq. (1) (*), with [A] set to the fixed agonist concentration, and basal, E_{max}, EC_{50}, and b set to the values obtained from the analysis of the agonist curve.

c. Using a Prism-type program:

i. Fit both curves to logistic functions; Eq. (2).

ii. Calculate K_b, the antagonist dissociation constant, by inserting the values of the fixed agonist concentration ([A]), the EC_{50} and slope *(b)* of the agonist fit and the IC_{50} from the antagonist fit, into the functional Cheng-Prusoff equation *(16)*.

$$K_b = \frac{[IC_{50}]}{\{2 + (\frac{[A]}{[EC_{50}]})^b\}^{1/b} - 1} \quad (3)$$

This analysis assumes that the antagonist Schild slope is 1.

10. If the agent does not affect basal activity, E_{max}, or the shape of the agonist curve, then the complete data set, together with the concentrations of agonist [A] and allosteric agent [X], are fitted to the equation:

$$\text{Effect} = \frac{E_{max} - \text{basal}}{1 + \{\frac{[EC_{50}]}{[A]} \cdot \frac{1 + [X]/K_X}{1 + \alpha \cdot [X]/K_X}\}^b} + \text{basal} \quad (4)\ (**)$$

to yield estimates of the dissociation constant of the allosteric agent, K_X, and the cooperativity with the agonist, α *(5)*.

11. Filters that are dried before addition of scintillation fluid can be counted immediately. Otherwise, it is important that all the water in the filter disk be dissolved in the scintillation fluid (*see* Note 7). ^3H is much more quenched by water then ^{35}S; if the filter disk is wet, the dual label program will not function correctly, and the results will be qualitatively incorrect.

12. Ideally, one isotope should not give more than about 30 times the counts given by the other isotope.

13. Two types of contamination may occur if a cell harvester is used with both [^{3}H]antagonist and [^{35}S]GTPγS.

a. The tubing will be contaminated with ^{35}S, which will leach out and contaminate ^3H assays. We have observed contamination of over 1000 dpm per sample. The contamination is progressively reduced during a day as more ^3H assays are filtered, but is higher at the start of the next day. We cope with the problem by routinely counting ^3H with a dual-label program.

b. Serious filter blank problems occur with [^{35}S]GTPγS if the filter is exposed to even very small amounts (1 part in 10^7 w/v) of polyethylenimine (PEI). In many radioligand binding assays, filters are soaked with 0.1% PEI to reduce the binding of positively charged radioligands. The filtration apparatus will become contaminated with PEI, and [^{35}S]GTPγS binding assays using the same apparatus will often have some spuriously high readings, especially with the first filter of the day. The solution is to filter washing buffer or water through two filters (or much cheaper, paper towels) before filtering [^{35}S]GTPγS.

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9

Autoradiographic Visualization in Brain of Receptor-G Protein Coupling Using [^{35}S]GTPγS Binding

Laura J. Sim, Dana E. Selley, and Steven R. Childers

1. Introduction

1.1. Autoradiographic Localization of Receptor-G Protein Coupling

Localization of receptors in brain sections using autoradiographic detection of radioligand binding has been an important technique in the neuroanatomical identification of a large number of neurotransmitter receptors. However, receptor autoradiography provides little information regarding the functional relevance of these sites and, in fact, does not establish which of the labeled receptor sites are actually coupled to intracellular signaling mechanisms. Fortunately, with the family of G protein-coupled receptors (GPCR), signal transduction is mediated at the level of the transducer itself (i.e., at the point at which receptors activate the α-subunits of G proteins to bind guanosine 5'-triphosphate [GTP]). The development of an assay for agonist-stimulated [^{35}S]guanylyl-5'-O-(γ-thio)-triphosphate ([^{35}S]GTPγS) binding, originally developed for receptors in isolated membranes, has provided an excellent opportunity to apply this process to brain sections, thus allowing the visualization of receptor-activated G proteins in specific brain regions.

This chapter describes the recent development of in vitro autoradiography of receptor-stimulated [^{35}S]GTPγS binding in brain sections. One of the keys to the success of this technique is the use of a large excess of guanosine 5'-diphosphate (GDP) to inactivate G protein α-subunits and reduce basal [^{35}S]GTPγS binding. Using this technique, [^{35}S]GTPγS binding, stimulated by multiple GPCRs, can be localized in adjacent sections using a single

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THE CYCLIC AMP SYSTEM AND DRUG DEVELOPMENT

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INTRODUCTION

A primary challenge to and a major goal of biochemists engaged in research on the development of new and useful drug substances has been the successful application of enzyme systems as screening tools for the detection of pharmacologically active agents. Over the years, enzyme systems involved in the biosynthesis of nucleic acids or proteins have been rather broadly studied in the quest for substances with anti-tumor or anti-viral activity (1). Although several interesting and useful compounds that inhibit these enzymatic processes have been found, they have, for the most part, proved to be toxic to the mammalian host in which they must be used because of their lack of tissue selectivity with respect to the inhibition of macromolecule synthesis (2). The inhibition of xanthine oxidase, an enzyme that is critically involved in the pathogenesis of gout, has led to the development of a useful therapeutic agent (allopurinol) for this disease (3). The significantly greater inhibition of dihydrofolic reductase of bacterial vs. mammalian cells by trimethoprim and the application thereof in the chemotherapy of infectious diseases are well known (4). Beyond these, very few examples exist of the successful development of useful therapeutic agents based on discovery or development of the activity in a defined enzyme system.

During the last few years, the important role played by the "cyclic AMP system" (for the purposes of this paper, this term will include the cyclase, phosphodiesterase, and kinase components of the system) in the metabolism of most, if not all, mammalian cells has made it eminently clear that perturbation of this ubiquitous regulatory mechanism could well lead to the discovery of agents with unique pharmacologic properties (5–7). The development of drugs via a search for either inhibitors or activators of the enzymes involved in cAMP metabolism and function is fraught with concern for specificity, since there is no way to predict *a priori* whether a given enzyme inhibitor will act in one tissue, in no tissue, or in all tissues, until it has been thoroughly studied *in vivo*. To this end, a program was initiated in our laboratories several years ago with the specific objective of discovering new chemical types that inhibited cAMP phosphodiesterase and, in addition,

showed some desirable pharmacologic property when tested in intact animals. The present paper describes some of the results obtained in this program.

INHIBITORS OF PHOSPHODIESTERASE (PDE)

Screening Results

To determine the general profiles of activity in inhibiting cAMP phosphodiesterase for a wide variety of substances, compounds known to have desirable pharmacologic activities or to be useful therapeutic agents, as well as new chemical entities available from our synthetic programs, were tested for their inhibition of PDE *in vitro* (8–10). Among 158 compounds representing 49 different classes of therapeutic agents, Weinryb and co-workers (10) reported some correlation between pharmacologic activity *in vivo* and inhibition of phosphodiesterase *in vitro*. Although such a finding obviously does

TABLE 1. INHIBITION OF PDE FROM DIFFERENT SOURCES BY SQ 20,009 OR THEOPHYLLINE

Source of Enzyme	I_{50} (μM)	
	SQ 20,009	Theophylline
Rat brain	2.0	120
Rabbit brain	4.8	150
Rat adrenal	20	130
Rat lipocyte	21	40
Cat heart	27	50

I_{50} is the concentration of compound causing 50% inhibition of enzyme activity *in vitro* (9).

not establish that the pharmacologic effects of these drugs in the animal are achieved by this mechanism of action, the results encourage the investigator to use the enzyme screen as a tool to detect a wide variety of agents with pharmacologic activities. Among the new compounds demonstrated to inhibit this enzyme, a family of pyrazolopyridines, synthesized at the Squibb International Research Center (11), was chosen for more detailed investigation, as described below.

Biochemical Studies With SQ 20,009

Chasin and his colleagues have described their studies on a new and potent inhibitor of PDE, designated SQ 20,009 (9). They reported that the I_{50} (concentration needed to inhibit cAMP phosphodiesterase by 50%) varied with the source of the enzyme being tested, as shown in Table 1.

It can be seen that a 10-fold difference in sensitivity to the effect of SQ 20,009 exists among PDEs from six sources and that SQ 20,009 was 60 times more potent than theophylline in inhibiting PDE from rat brain. Dose-response curves for SQ 20,009, theophylline, and caffeine against rat brain PDE are shown in Figure 1, and the double-reciprocal plot showing the competitive nature of the inhibitory action is presented in Figure 2. From these data, it is clear that SQ 20,009 is a potent inhibitor of cyclic phosphodiesterase and, being a new chemical type, it warranted, in our view, extensive pharmacologic screening to determine whether it possessed desirable pharmacologic properties.

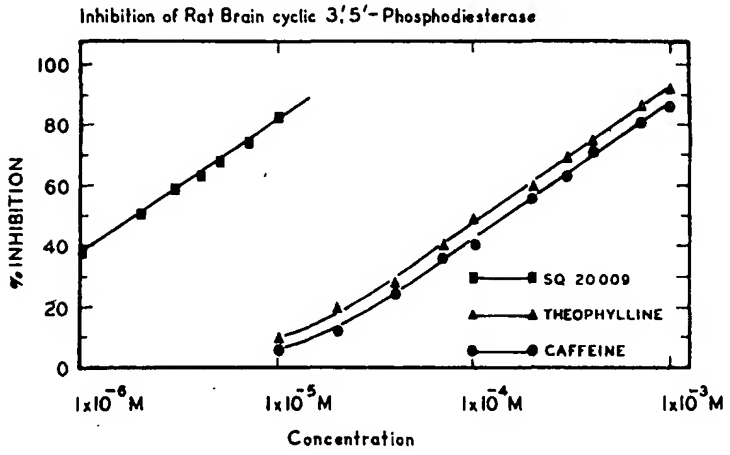


FIG. 1. Inhibition of rat-brain cyclic 3'-5'-AMP phosphodiesterase by SQ 20009, theophylline, and caffeine. Assays were performed as described in Ref. 9. Each point represents a single determination.

Pharmacologic Studies With SQ 20,009

In initial screening procedures in the rat, SQ 20,009 was found to be active in the CNS area and more detailed investigations disclosed significant anxiolytic activity for this compound (12, 13), as shown in Figure 3. The results of comparative investigations of selected reference compounds are presented in Figures 3 and 4.

These data show that anti-anxiety activity, at least in the rodent, is observable after treatment of the animals with inhibitors of PDE and with cAMP itself. Whether the mechanism of action of compounds like SQ 20,009 relates to this biochemical effect has not been established. Recent studies in our laboratories failed to show an increase in the concentration of cAMP in

brain tissue after the administration of SQ 20,009, as determined by a modified radioimmunoassay for the cyclic nucleotide (14). Although this finding could mean that SQ 20,009 exerts its anxiolytic activity in animals by a mechanism other than the inhibition of phosphodiesterase, it might also be explained in other ways. For example, alteration of the cAMP concentration in glial cells might result in a change in the opposite direction in the concentration of cAMP in neurons, as was found to be the case with certain changes in nucleic acid patterns (15). Alternatively, it could mean that the change occurred in a very small segment of brain tissue and was simply not detectable

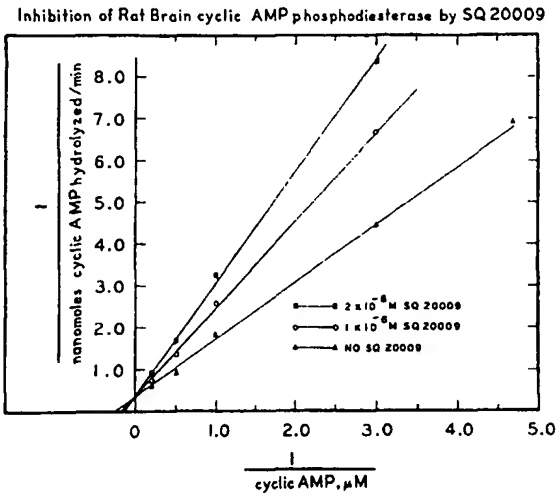


FIG. 2. Double-reciprocal plot of the hydrolysis of cyclic AMP by the low K_m rat-brain cyclic AMP phosphodiesterase and the inhibition of this hydrolysis by SQ 20009. Assays were performed as described in Ref. 9. Each point represents the average of 6 determinations. Due to limitations of space, a number of points on each line have been omitted from the figure. The equations of the lines were calculated by computer for all experimental points.

in the specimen taken for assay. It is also possible that the biological activity is due to a change in the ratio of cAMP to cGMP (16), or that a *very* small change in the concentration of cAMP is sufficient to induce the CNS change observed, as has been reported for the adrenal cell (17). Still another possible explanation is the release of a cyclase inhibitor (18) or increase in phosphodiesterase activity (19) in the cell subsequent to a small increase in cAMP *per se*. These speculations can be confirmed or negated only by further laboratory experimentation.

In spite of our inability to show a clear correlation between an increased level of cAMP in the brain and the anxiolytic activity of SQ 20,009, a statistically significant relationship between these effects has been demonstrated for other compounds that inhibit PDE *in vitro* and show anxiolytic activity in

animals (12), as shown in Table 2. Of those compounds able to inhibit brain PDE *in vitro* at a concentration below 1000 mM, 11 of 15 were active in the rat as anxiolytic agents. Of the four that were not active, two (amitriptyline and nortriptyline) are known antidepressive agents of the tricyclic type. On the other hand, imipramine, also a tricyclic antidepressive, did show activity

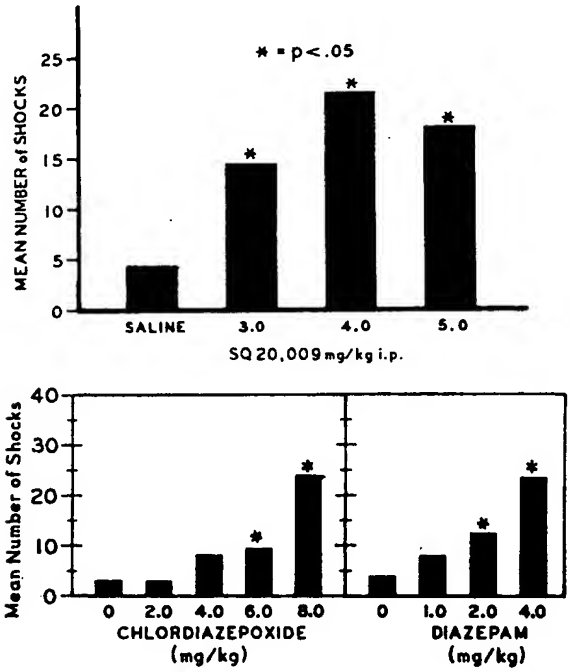


FIG. 3. Mean number of shocks received by rats during the 3-min test session—after intraperitoneal administration of SQ 20009, chlordiazepoxide, or diazepam (12).

in both the enzyme and anxiolytic assays. Meprobamate, pentobarbital, and phenobarbital, none of which showed significant inhibition of the enzyme *in vitro*, were all active in the conflict test. As is the case with SQ 20,009, it cannot be stated from the data available to date, whether or not the correlation observed is the result of inhibition of PDE activity in the whole animal.

Specificity of Action

Specificity of action for one (or a few) of all the tissues of the body that could conceivably be affected by an inhibitor of cAMP phosphodiesterase is a *sine qua non* for the development of a truly useful drug substance. Based

on studies with SQ 20,009 *in vitro*, one should have been quite pessimistic about being able to administer this compound to an animal without activating several biological systems in the body. For example, SQ 20,009 stimulated lipolysis and steroidogenesis in the presence of suitable agonists (e.g., epinephrine, ACTH, or cAMP) as illustrated in Figure 5 (20). In the lipocyte, theophylline was somewhat more potent than SQ 20,009, whereas the reverse

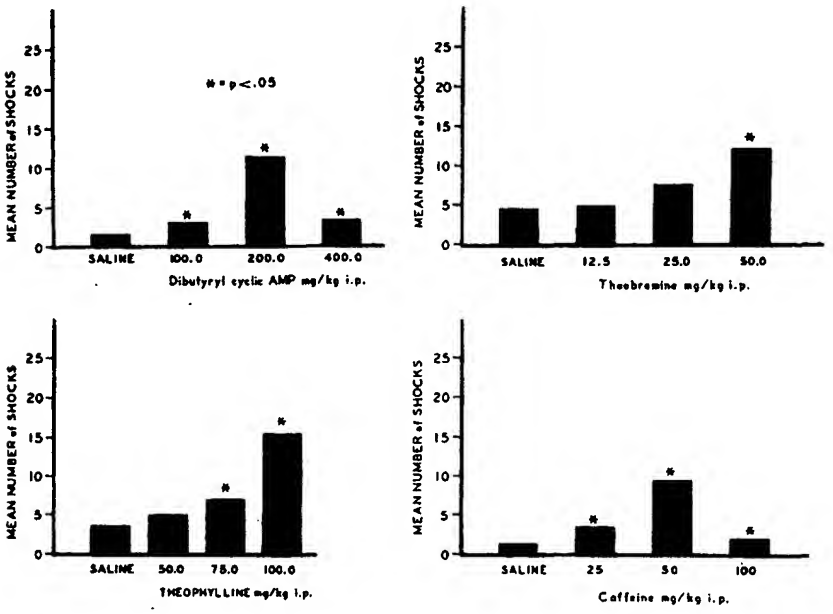


FIG. 4. Mean number of shocks in the 3-min test session—after intraperitoneal administration of dibutyryl cyclic AMP, theobromine, theophylline, or caffeine to rats (12, 13).

was true for the adrenal cell preparation. When SQ 20,009 was tested *in vitro*, it was shown to stimulate the release of insulin from the pancreas of the fetal rat, in the presence and absence of 5.5 mM glucose (Renold, A. E., personal communication, to be published). At the high glucose concentration, SQ 20,009 was 50 to 100 times more potent than theophylline; in the absence of added glucose, the effects were qualitatively different (i.e., theophylline did not stimulate the release of insulin in the absence of glucose). In the isolated pancreas of the golden hamster, SQ 20,009 was 25 to 50 times more potent than theophylline in promoting insulin release and, as in the fetal rat preparation, differed from theophylline in being able to do so at both low (0.6 mg/ml) and high (3 mg/ml) glucose concentrations (Lebovitz, H., personal communication, to be published).

TABLE 2. RELATION BETWEEN THE EFFECTIVENESS OF A DRUG IN THE RAT CONFLICT TEST AND ITS ABILITY TO INHIBIT CYCLIC AMP PHOSPHODIESTERASE ACTIVITY IN VITRO (12)

Drug	I_{50}	Conflict score*	P
Section A			
SQ 20,009	2	8.0	<.002
Diazepam	33	12.5	<.006
Fluphenazine	48	2.7	<.01
Chlordiazepoxide	110	8.3	<.001
Theophylline	120	3.0	<.02
Caffeine	150	4.8	<.003
Theobromine	150	2.7	<.02
Chlorpromazine	170	2.5	<.02
Reserpine	170	0.6	>.05
Thioridizine	180	4.6	<.05
Nortriptyline	200	0.9	>.05
Trifluprομazine	300	1.9	<.02
Amitriptyline	460	0.8	>.05
Diphenylhydantoin	480	0.7	>.05
Imipramine	700	1.8	<.05
Section B			
Meprobamate	>1000	36.0	<.001
Pentobarbital	>1000	13.5	<.001
Parachlorophenylalanine	>1000	1.9	>.05
Phenobarbital	>1000	1.9	<.02
Haloperidol	>1000	1.6	<.04
Morphine	>1000	1.4	>.05
Atropine	>1000	1.1	>.05
Chloral hydrate	>1000	1.1	>.05
Amphetamine	>1000	0.9	>.05
Doxepin	>1000	0.7	<.01
Procaine amide	>1000	0.5	>.05
Codeine	>1000	0.3	>.05
Methylphenidate	>1000	0.1	>.05
Scopolamine	>1000	0.1	>.05

* Conflict score = $(N_{max}/N_{CDP}) \times (N_{max}/N_{cont.ol})$; where N_{max} is the maximum number of shocks after drug administration; N_{CDP} is the number of shocks after chlordiazepoxide administration; $N_{control}$ is the number of shocks after the control injection. All numbers are expressed as means.

In spite of the findings *in vitro*, when SQ 20,009 was administered orally for 4 to 5 weeks to dogs and monkeys at repeated daily doses as large as 50 and 25 mg/kg, respectively, no effects were observed on homeostatic systems, as indicated by tests that included the determinations in blood of glucose, insulin, triiodothyronine (T_3), free fatty acids (FFA), cortisol, and calcium, and complete urinalyses that included the excretions of calcium

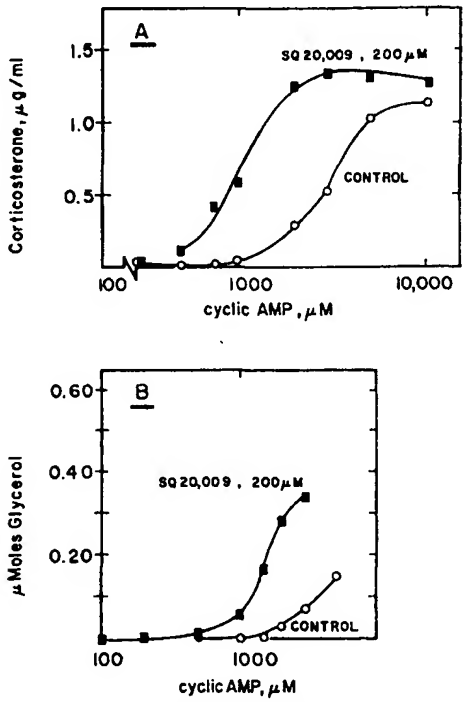


FIG. 5. Effect of SQ 20009 on cAMP-induced lipolysis and steroidogenesis (19).

and 17-ketosteroids. In view of the striking effects observed *in vitro* in the pancreas of the fetal rat, SQ 20,009 was infused (15 mg/kg) into the pancreatic artery in each of five dogs during a 1-hr period; under these conditions, there was a significant increase in concentrations of insulin (to 150% of baseline) and glucose (20% over baseline) in the blood (Poutsiaka, J. W. *et al.*, unpublished observations). This experiment demonstrated that SQ 20,009 *can* effect insulin release *in vivo* when it is administered directly to the pancreas; it fails to do so when administered orally or through a peripheral vein, either because the concentration of unchanged drug (or specific, biologically active metabolite) that reaches the pancreas is insufficient or the rate at which it enters the pancreas is insufficient to effect the release of insulin.

Whatever the reason, no change in insulin-glucose balance was seen in animals of several species that were showing definite CNS effects following SQ 20,009, illustrating the striking specificity of action of this compound in the intact animal. In clinical trials, the oral administration of SQ 20,009 using different dosage regimens to a maximum of 250 mg as a single dose or 50 mg four times daily, also failed to show any significant change in a wide variety of parameters (including insulin, T_3, tetraiodothyronine, testosterone, cortisol, FFA, glycerol, and glucose) although clear evidence of effects on the CNS system was again observed (Neiss, E. *et al.*, unpublished observations).

To explore further the question of selectivity of action, extensive structure-activity studies were conducted in our laboratories. Selected examples of the changes in biological activity observed in cellular test systems and in intact animals for analogs of SQ 20,009 that showed significant inhibitory activity against brain PDE are shown in Table 3. These data illustrate clearly marked differences in biological profile for compounds that are chemically related to one another and that inhibit PDE *in vitro*. These differences can be explained in a variety of ways. It is highly probable that penetration into different types of tissues varies with the compound under investigation. Likewise, the metabolic pattern in animals, the rate of absorption from the gut and distribution to specific tissue sites in the body is also likely to vary, possibly quite considerably. The activity of the compounds against the enzymes from different tissues can also vary and, coupled with difference in rate of penetration, can lead to *very* significant differences of activity in intact animals, such as those observed. Whatever the reason (or reasons), it is clear that one cannot predict *a priori*, at least for this family of drugs, that an analog of a compound with known activities *in vitro* and in intact animals will reproduce those same activities *in vivo*, even though its ability to inhibit the enzyme *in vitro* is comparable to that of the parent compound.

INHIBITORS OR ACTIVATORS OF ADENYL CYCLASE

In addition to searching for drugs that alter the activity of the phosphodiesterase system, one might search for those that affect adenyl cyclase which catalyzes the synthesis of cAMP in the cell. Indeed, it has been suggested that the β-receptor site is contiguous with, and perhaps identical to, the enzyme site of adenylate cyclase and it has been postulated, accordingly, that many varied agents with physiological or pharmacologic actions exert their effects by activating or inhibiting this enzyme (5, 21, 22). Because it is inherently more difficult to assay quantitatively for adenyl cyclase activity than for phosphodiesterase activity, the search for new agents that inhibit or stimulate this enzyme necessarily proceeds much more slowly. In the assay

TABLE 3. SPECIFICITY OF ACTION OF ANALOGS OF SQ 20,009

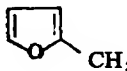
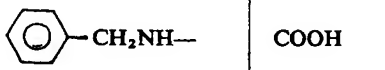
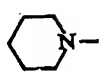
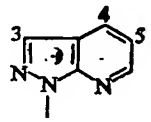
Structure*					Enzyme activity†				
SQ	R_1	R_3	R_4	R_5	Brain PDE	Heart PDE	Lipolysis	Adrenal steroidogenesis	Pharmacologic profile
20,009	C_2H_5	H	CH_3 \ C=N—NH— / CH_3	$COOC_2H_5$	3	2	2	3	Marked CNS activity
65,442	C_2H_5	H	C_2H_5S—	$COOC_2H_5$	3	2	3	2	No pronounced activity
66,110	H	CH_3	*n*-C_4H_9NH—	$COOC_2H_5$	3	3	3	3	No pronounced activity
66,444		CH_3	*n*-C_4H_9NH—	$COOC_2H_5$	3	0	3	3	No pronounced activity
66,093	C_2H_5	H	$CH_2=CH—CH_2NH$—	$COOC_2H_5$	3	2	2	1	Hypoglycemic; diuretic
66,596	C_2H_5	H		COOH	0	2	3	1	No pronounced activity
66,109	C_2H_5	H	CH_3CH_2CH—NH— | CH_3	CO— 	3	0	3	0	No pronounced activity
66,438	C_2H_5	H	CH_3CH_2CH—NH— | CH_3	COC_4H_9	3	1	3	1	Slight CNS activity; anti-inflammatory

TABLE 3—(*continued*)

66,571	C_2H_5	CH_3		$COOC_2H_5$	3	0	0	NT	Anti-inflammatory

* 4-Substituted pyrazolopyridines 

† Rating scale in which 1 represents an ID_{50} in the range of 50–100 μM and 3 is an $ID_{50} < 10\ \mu M$ for the PDE preparations; for the lipocyte system, a rating of 3 represents > 100% stimulation while a rating of 1 = 10–40% stimulation.

system used in our laboratories, compounds known to stimulate cyclase (isoproterenol, epinephrine, norepinephrine, fluoride, and prostaglandin) caused an increase of 40 to 400% of basal activity (23). When approximately 50 purine bases, nucleosides, nucleotides (both normal and abnormal) and cyclic nucleotides were tested for inhibition in this system, several were active at concentrations less than 1 mM, as shown in Table 4. The 2'O-palmitoyl derivative of cAMP was the most active compound found in this series. There is no reason *a priori* to have selected this substance as an inhibitor of adenyl cyclase nor is there any explanation at the moment of its molecular mechanism of action. Kinetic studies with this compound show a Michaelis constant (K_m) for the conversion of ATP to cAMP of 0.17 to

TABLE 4. INHIBITION OF ADENYL CYCLASE FROM GUINEA PIG LUNG

Compound	I_{50}* (mM)
Inosine monophosphate (IMP)	1.0
8-thiohypoxanthine	0.5
6-thiopurine	0.5
8-bromo cyclic AMP	0.8
6-ethylthiopurine cyclic ribotide	1.0
8-thio cyclic IMP	0.6
N^6, 2'O-dipalmitoyl cyclic AMP	0.4
2'O-palmitoyl cyclic AMP	0.03

* Concentration that results in 50% inhibition of enzyme activity (23).

0.32 mM, based on three different enzyme preparations. Inhibition appeared to be competitive, with a K_i of 0.01 mM (23). Pharmacologic testing of this ester showed no significant activity *in vivo*, although antagonism of prostaglandin action on smooth muscle was observed as presented in Table 5. No overt pharmacologic action of a desirable nature has been found as yet with palmitoyl cAMP, but further studies aimed at determining whether selective effects can be achieved in intact animals are currently underway in our laboratories.

ANALOGS AND DERIVATIVES OF CYCLIC AMP

A third leg of the triangle that constitutes the "cyclic AMP system" is the direct action of cAMP *per se* on the kinases that it activates and on possible feedback mechanisms that affect adenyl cyclase. A wide variety of cyclic nucleotides (24, 25) have been evaluated in our laboratories, both biochemically and pharmacologically. Many naturally occurring and sub-

stituted cyclic nucleotides and derivatives thereof were shown to be inhibitors of the phosphodiesterase (26), and were also found to produce steroidogenic effects, lipolytic effects, or both, in isolated, whole-cell preparations *in vitro* (27). Selected examples are presented in Table 6. These results again show unpredictable and, perhaps, unexpected specificities, most of which cannot

TABLE 5. PHARMACOLOGIC PROFILE OF 2'O-PALMITOYL CYCLIC AMP

Test System	Dose *in vivo* (mg/kg, i.p.) or Concentration *in vitro* (μg/ml)	Agonist	Result
Intact rat	200	—	Toxic
CNS screen	100	—	Noapparent effect
Spontaneously hypertensive rat	50	—	No effect on blood pressure
Contraction of smooth muscle *in vitro*			
Rat portal vein			
Stimulation or blocking of α-adrenergic receptors	128	Dopamine	No effect
Stimulation or blocking of β-adrenergic receptors	128	Isoproterenol	No effect
Rat colon	128	Angiotensin	No effect
Guinea pig ileum	128	Acetylcholine	No effect
	128	Histamine	No effect
	128	None	No effect
	80	Barium chloride	EC_{50}
	35	Bradykinin	EC_{50}
	5	PGE	EC_{50}
Whole cell systems *in vitro*			
Inhibition of fat cell lipolysis	25	Epinephrine	I_{50}
	200	cAMP	I_{50}
Inhibition of adrenal cell steroidogenesis	>100	ACTH	I_{50}

EC_{50} = conc. inhibiting contractile response by 50%; I_{50} = concentration causing 50% inhibition of response.

yet be explained. Unlike the pyrazolopyridine inhibitors discussed earlier, the analogs of cAMP are generally more active in inhibiting cat heart PDE than in inhibiting rat brain PDE. In the whole-cell preparations, on the contrary, diverse effects are seen, including: (1) good stimulation of steroidogenesis and lipolysis by a rather potent inhibitor of PDE and activator of protein kinase

(8-thiomethyl cAMP); (2) significant activity in both cellular preparations by a compound that is essentially inactive as a PDE inhibitor at the enzyme level (8-hydroxy cAMP) although active on the kinase; and (3) a reversal in the expected correlation of activity in the adrenal-cell preparation with that in the enzyme preparation (8-monomethylamino and 8-dimethylamino derivatives of cAMP). When one considers the probable differences in rate of penetration through different membranes, as well as possibilities for metabolic interconversions within different cells, these results are not particularly surprising. The effects could be based, for example, on better penetration of the adrenal cell by the N-dimethylamino compound than by the mono-

TABLE 6. ACTIVITIES OF CYCLIC NUCLEOTIDES IN VITRO

Compound	I_{50}* (μM)		A_{50} (μM)†		Kinase‡ Stimulation
	Cat Heart PDE	Rat Brain PDE	Adrenal	Lipocyte	
cAMP	—	—	3300	8500	1.0
Dibutyryl cAMP	100	650	95	500	—
8-SH-cAMP	330	540	380	250	2.0
8-SCH_3-cAMP	39	125	65	180	2.4
8-SC_2H_5-cAMP	41	52	110	230	0.9
8-Br-cAMP	16	67	85	440	0.7
8-OH-cAMP	$>10^{-2}$ M	$>10^{-2}$ M	90	260	1.0
8-NH_2-cAMP	23	400	150	1200	1.7
8-$NH(CH_3)$-cAMP	160	700	460	3300	0.8
8-$N(CH_3)_2$-cAMP	3300	2300	130	3000	0.4

* Concentration *in vitro* that causes 50% inhibition of enzyme activity.

† Concentration *in vitro* that causes half-maximal steroidogenesis of lipolysis under the conditions employed (see ref. 26 for details of methods).

‡ Relative activity as activators of bovine brain kinase at 10^{-6} M (24).

methylamino derivative, with subsequent conversion of the dimethylamino to the monomethylamino or free amino compound within the cell. Alternatively, the apparent disparity between enzyme inhibition and effects in whole cells may be the result of the compound acting like cAMP and directly activating the kinase after dealkylation within the cell, perhaps with concomitant activation of the kinase and inhibition of PDE. No definite answers can be given until further experiments have been performed.

In the case of certain of the 8-substituted cAMP analogs, it was of considerable interest to find that this type of compound, administered intravenously to dogs, stimulated a hyperglycemic response, with corresponding changes in plasmic concentrations of glucagon and insulin, but without apparent effect on other endocrine systems. Illustrative of these findings are the experiments with 8-thiomethyl cAMP. Although this compound (1)

caused significant inhibition of PDE from either cat heart or rat brain, (2) activated protein kinase at low concentration and (3) stimulated both lipolysis and steroidogenesis in isolated whole-cell preparations *in vitro*, it did not cause CNS or cardiovascular changes when administered intravenously to dogs at doses of 2.5 mg/kg daily for 4 weeks. Doses of 8-thiomethyl cAMP as large as 4.5 mg/kg, given to anesthetized dogs by slow intravenous infusion caused no changes in blood concentration of cortisol, glycerol, glutamic-pyruvic transaminase, or urea nitrogen, nor in pH, pO_2, or pCO_2 (Poutsiaka, J. W. *et al.*, unpublished observations). On the other hand, like glucagon, it caused a significant increase in concentration of blood glucose ($\sim$100% over baseline) at doses as low as 0.15 mg/kg, perhaps by increasing the rate of glycogenolysis in the liver.

DISCUSSION

The data presented in this paper illustrate the value of using the enzymes that affect the formation and metabolism of cAMP as tools in the search for potential new drug substances. New chemical types and new derivatives within known chemical families have been found to be biologically active through the use of such screening procedures, not only in our laboratories, but in others that employ a similar approach (28–30). There can be little doubt that sufficient selectivity of action in whole animalscan be demonstrated for compounds that perturb the cAMP system to make this approach a valuable one in the search for drugs. Unfortunately, it is not possible to predict precisely which compounds will be inhibitors or in which tissues they will work in an intact animal, even after extensive investigations *in vitro* with isolated-cell systems and tissue preparations. This shortcoming, we believe, is a result of the multitude of factors that must come into play with every drug. For example, possibilities for metabolic or chemical conversions exist in the acid milieu of the stomach, during exposure to enzymes in the intestine, during passage through the intestinal tract, in the liver and, in some cases, in particular tissues in which the compound finally resides. In addition, different compounds may be subject to marked differences in absorption, not only from the intestine, but also from the particular sites where the action of those compounds will be displayed. One might conclude, after considering all these factors, that *no* possibility exists for developing into a useful drug any substance, other than one that is totally absorbed, is not metabolized, and acts on only one tissue *in vivo*. The history of drug development tells us clearly that such is usually not the case. Indeed, many, if not most, valuable drugs are metabolized by the host, and the absorption and distribution among various tissues usually does *not* explain the observed selectivity of effect. Nevertheless, we believe that a screen that tests the effects of compounds on enzymes that are likely to be involved in some disease state will lead us to

new and useful drug substances with unique pharmacologic profiles. The challenge is great, as is the amount of work needed to accomplish the desired result!

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MINIREVIEW

POSSIBILITIES FOR DRUG DEVELOPMENT BASED ON THE CYCLIC AMP SYSTEM

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It is increasingly apparent to those involved in drug research and development that it is becoming more difficult to produce new drug entities (1)*. It is also evident that it is no longer efficient to develop drugs primarily by mass synthesis and screening. Drug development must give way to more sophisticated and effective means of producing useful entities, based on a thorough knowledge of the biochemical and pathophysiologic lesion(s) of the disease state for which the drug is designed (2).

The discovery of cyclic adenosine 3',5'-monophosphate (cyclic AMP) by Earl Sutherland and Theodore Rall and the elucidation of its role in cellular function provide a great opportunity to use this system as a means for the development of new drugs. Cyclic AMP acts not only as a second messenger for the effects of nearly all hormones, but also, together with the other known natural cyclic nucleotide, cyclic guanosine 3',5'-monophosphate (cyclic GMP), appears to be intimately involved in the control of almost all facets of cellular activity (3-7). Abnormal levels of cyclic AMP and/or cyclic GMP are also associated with a number of disease states most notably cancer, hypertension, asthma, cholera, diabetes, a large number of endocrinopathies and possibly aging. It is therefore quite possible that drugs designed to modify the cyclic nucleotide system might, at least theoretically, be of value in the

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* The references cited in this article are not complete and were selected to represent others dealing with similar points.

treatment of these conditions. It is the purpose of this minireview to examine the possible ways by which changes in the cyclic nucleotide system can be achieved to secure possible means for treating disease.

Agents that alter the cyclic nucleotide system may be classified as follows:

I. AGENTS THAT ALTER THE INTRACELLULAR LEVELS OF THE CYCLIC NUCLEOTIDES BY AFFECTING THEIR
   - A. SYNTHESIS
   - B. LOSS TO EXTRACELLULAR FLUID, AND
   - C. DEGRADATION.

II. AGENTS THAT ALTER THE INTRACELLULAR ACTIVITY OF THE CYCLIC NUCLEOTIDES BY CHANGING
   - A. THE SIZE OF THEIR FREE POOL,
   - B. MIMICKING THEIR ACTIONS, AND
   - C. ANTAGONIZING THEIR INTRACELLULAR EFFECTS.

III. AGENTS THAT AFFECT THE CYCLIC NUCLEOTIDE SYSTEM SECONDARY TO THEIR EFFECTS ELSEWHERE INCLUDE
   - A. PROSTAGLANDINS, THEIR DERIVATIVES AND ANTAGONISTS; AND
   - B. AGENTS AFFECTING CATION AVAILABILITY; AND
   - C. OTHERS

These classes will be discussed separately:

I. AGENTS ALTERING THE INTRACELLULAR LEVELS OF THE CYCLIC NUCLEOTIDES:

A. DRUGS THAT COULD AFFECT THE SYNTHESIS OF THE CYCLIC NUCLEOTIDES: Specific cyclases appear to catalyze the synthesis of either cyclic nucleotide. Adenylyl cyclase (AC), which controls the rate of synthesis of cyclic AMP from ATP, is a membrane-bound enzyme that requires Mg^{++} for activity and is stimulated by a large variety of hormones. In contrast to the wealth of information about AC, comparatively little is known about guanylyl cyclase (GC), the enzyme catalyzing the synthesis of cyclic GMP from GTP. It

is clear that GC activity resides in both the particulate and soluble fractions of cells and appears to be entirely dependent on the presence of Mn^{++}. Calcium ions are also of paramount importance for the activity of GC in intact cells. GC is also stimulated by a variety of hormones. The two cyclases are interrelated in at least two ways: (a) GTP, the substrate for GC, is a cofactor for the activity of AC in at least some systems (8) and (b) Ca^{++} ions increase the activity of GC and at the same time inhibit the activity of AC.

From the point of view of drugs, apart from the poorly understood stimulatory effects of fluoride on AC activity, stimulation of AC and GC has thus far been sacrosanct to only hormones or their closely related derivatives. No other compound(s) not included in that class was shown to stimulate either AC or GC. This may be due to the highly specific discriminative nature of these cyclases which appear to be primarily designed to distinguish only the natural information transferring molecules (hormones). Only those ligands with the strict structural requirements are able to stimulate the cyclases. One possible exception is a family of bacterial toxins including cholera and *E. coli*. Therefore, it is rather unlikely that drugs other than hormonal relatives could be produced that act via stimulation of the activity of either cyclase. A possible drug action however, is the specific blockade of the effects of the hormones on AC or GC; for example, β-adrenergic receptor blockers do block the stimulation of AC by β-adrenergic receptor stimulants. The tissue specificity of AC makes it possible to obtain tissue selective β-adrenergic blockade (9). Similarly, atropine blocks the stimulatory effects of Ach on GC. The nature of the chemical structures that are capable of blocking the stimulation of AC by protein hormones is presently unknown. A peptide derivative of oxytocin seems to block its AC-stimulatory effects in rabbit kidney (10). This may present an area for future drug development.

B. DRUGS THAT COULD AFFECT CYCLIC NUCLEOTIDE LOSS TO EXTRACELLULAR FLUID: Loss of cyclic AMP and/or cyclic GMP to the extracellular fluid is now recognized as an important factor in determining the intracellular

levels of the cyclic nucleotides. It is not clear whether this process is active or passive. Cyclic nucleotides leaking outside the cell appear to mirror and can sometimes serve as a reliable indicator of their intracellular concentrations. It is not known whether this process can be affected by either hormones or drugs. Although it is too early to judge the possibilities of this process in drug development, it remains potentially important.

C. DRUGS THAT COULD AFFECT THE DEGRADATION OF THE CYCLIC NUCLEOTIDES: The hydrolysis of cyclic AMP and/or cyclic GMP by their respective phosphodiesterases (PDE's) appears to provide a great possibility for altering the intracellular levels of the cyclic nucleotides via the use of drugs. For a compound to be useful as a drug acting via its PDE effects, certain requirements should be met.

1. ACTIVITY. The drug should be active on the enzyme (either stimulate or inhibit) in reasonable levels. Furthermore, it should be capable of reaching its site of action (smooth muscle, brain, heart, etc.) in effective concentrations.

2. SELECTIVITY. PDE's are ubiquitous and are present in nearly all mammalian cells. A compound that acts uniformly on all PDE's would show little or no selectivity and would be of little value as a drug. Thus, for the compound to be useful it must show selectivity either in reaching its site of action (*e.g.*, ability to cross the blood brain barrier, selective uptake by a certain tissue, etc.) or in its specific PDE activity (*i.e.*, the drug should act on PDE of certain tissues only). Since these PDE's appear to exist in different forms, an intimate knowledge of the importance and properties of the two forms is essential for possible drug development. The different forms of PDE include:

a. Different forms in the same cell line. It is now well established that at least two, possibly inter-convertible, forms of the enzyme exist in different tissues. The two forms differ not only in their Km values for the substrate, but also in their heat and drug sensitivities, response to divalent cations, substrate specificity, electrophoretic and chromatographic behavior

and subcellular localization. The low Km form is apparently the more important form since its Km value is in the range of the *in vivo* levels of the cyclic nucleotides. There is an increasing number of reports describing more than two forms of PDE in a number of tissues. In most of these cases, however, the multiple forms could be traced to multiple cell types in the preparations used. A case in point is the studies of Weiss and associates who have shown at least six forms of the enzyme (11) in rat brain. However, when homologous preparations of rat astrocytoma cells were examined, only two forms were found (12).

From the point of view of drug development, the sensitivities of the two forms of PDE's for different stimulators or inhibitors appear to be different (13,14). This is a very important point since it is doubtful that agents acting only on the high Km forms of the enzyme would be useful. It appears that screening compounds for possible activity against PDE should stress the importance of the low Km forms since these are more likely to be the more important.

b. Different phosphodiesterases in different cell lines. Recent evidence also indicates that there are some differences in the PDE's from different tissues. In addition to possible structural and kinetic differences there also seem to be some differences in their responses to drugs (15,16). PDE screening methods based on enzymes isolated from a variety of tissues would appear to be a valid approach toward the empirical determination of tissue selectivity of the compounds in question.

c. Cyclic AMP *vs.* Cyclic GMP phosphodiesterases. It is believed that the two naturally-occuring cyclic nucleotides are hydrolyzed by similar sets of enzymes. Increasing evidence indicates that at least some selectivity towards either nucleotide may exist (17,18). Whether these differences are of sufficient magnitude to permit selective effects of these drugs on the hydrolysis of one or the other cyclic nucleotide remains to be seen (19). This is further complicated by the apparent ability of either nucleotide to

influence the rate of hydrolysis of the other (20-23). Thus, inhibition of the hydrolysis of either cyclic nucleotide resulting in a rise in its intracellular concentration will undoubtedly affect the rate of hydrolysis of the other and consequently its intracellular concentration.

There is increasing evidence for the possibly antagonistic roles of the two cyclic nucleotides in the control of cellular function, at least in some systems. It is therefore of paramount significance if selective effects on either PDE are to be achieved.

Other aspects essential to the development of drugs acting via PDE may include:

i) Agents that can modify PDE activity. The activity of the cyclic nucleotide PDE appears, at least in some tissues, to be under the influence of activators and/or inhibitors. It is conceivable that compounds could be found that interfere with the normal activation or inhibition of these enzymes with pronounced effects on their activity. No such compounds are yet available however.

Several compounds appear to affect PDE activity directly. By far the most frequent effect on PDE is inhibition. Only imidazole, some of its close relatives, including histamine (24), and some bitter tasting agents (25) were reported to stimulate the enzyme.

Some hormones also appear to affect PDE activity. These include insulin, cholecystokinin and other gastrointestinal hormones with a similar C-terminal amino acid sequence (26,27), thyroxin (28) and possibly epinephrine (29,30). It is therefore possible that compounds could be found that, although devoid of intrinsic activity on PDE, may block or facilitate the effects of these hormones on the enzyme.

ii) Diseases where drugs acting on phosphodiesterase are most desirable. In some diseases, the lowered cyclic AMP levels are associated with decreased sensitivity of AC to stimulation. The only possible approach to correct the cyclic AMP deficiency under these circumstances is the inhibition

of PDE. Examples of these condions are hypertension (31,32), asthma (33), psoriasis (34) and possibly cancer (35,36).

In some situations as in hypertension (31), hepatomas (37) and diabetes (38) increased activity of the low Km (high affinity) PDE may be an important part of the etiology of disease state. In these conditions, the use of PDE inhibitors would alleviate the deficiency in intracellular cyclic AMP by the most desired mechanism for any drug to act: namely, by correcting the basic biological defect.

iii) Drugs known to act via their effects on PDE. There are a number of known drugs that may owe their desirable activity to their effects on PDE (39). Examples of these are papaverine (40-43), sulphonylureas (44,45), diazoxide (46,47), morphine (48,49), chlorpromazine (50,51), thiazides (52,53), tricyclic antidepressants (54), reserpine (51), nicotinamide (55) and theophylline (53) in addition to a number of others (56-62). The possibilities for structure-activity relationships are quite unlimited. Newer classes of compounds will undoubtedly be added to the list of those that can affect PDE activity with possibly greater tissue and nucleotide selectivity.

II. AGENTS ALTERING THE INTRACELLULAR ACTIVITY OF CYCLIC NUCLEOTIDES:

A. AGENTS THAT AFFECT THE FREE-POOL SIZE: Intracellularly, the cyclic nucleotides appear to exist in at least two functional pools that respond differently to different agents (63). Cyclic GMP and cyclic AMP may also be localized in different parts of the same cell (64). The two cyclic nucleotides probably exist intracellularly in a sequestered state generally unavailable for control function since their total intracellular levels in many systems exceed the maximal effective concentrations on their target enzymes, *e.g.*, cyclic nucleotide-dependent protein kinases. If the cyclic nucleotides were freely accessible intracellularly, maximal activation of these target enzymes would occur and it would be impossible for these nucleotides to exert

any control function. Hence, it is assumed that these agents are somehow sequestered or bound intracellularly, probably to specific binding proteins, and are made available only when the need arises. It is conceivable that agents could be found that can interfere with the binding or sequestration of the cyclic nucleotides. Such agents would be capable of increasing the effective free intracellular levels of the nucleotides, with a resultant augmentation of their intracellular effects, without affecting their total concentration.

B. DRUGS THAT MIMIC THE ACTIONS OF THE CYCLIC NUCLEOTIDES: Derivatives or analogs of the cyclic nucleotides can mimic the effects of the natural cyclic nucleotides and thus supplement their effects. This could be achieved by either (a) increasing penetration into cells, since the naturally occurring cyclic nucleotides are notoriously unable to do so, or (b) having a greater intrinsic ability to activate the enzyme systems normally activated by the cyclic nucleotides. These effects may be useful in certain endocrinopathies and other other conditions where the levels of the cyclic nucleotides are low, *e.g.*, cyclic AMP in cancer.

C. DRUGS THAT ANTAGONIZE THE INTRACELLULAR EFFECTS OF THE CYCLIC NUCLEOTIDES: Derivatives of the cyclic nucleotides could, at least theoretically, antagonize the effects of the naturally occurring cyclic nucleotides on their specific target enzymes. Such agents would be useful in situations where excessive cyclic nucleotide levels are present, *e.g.*,cyclic AMP in diabetes, alcoholism (65), cholera (66) and mania (67) or cyclic GMP in psoriasis (68), and possibly hypertension. There is some evidence that tolbuamide acts in adipose tissue by a similar mechanism (69).

Some naturally-occurring cyclic nucleotide antagonists that are capable of modulating the effects of the cyclic nucleotides are known. Two such antagonists have been described: (a) a heat-sensitive protein-like material in leukocytes (70) and in rabbit skeletal muscle (71), and (b) a heat-stable, low molecular weight substance (72). The determination of the chemical structure

of the latter antagonist would be an extremely important development and may in fact open the door for an entirely new class of drugs with a novel mode of action.

Conceivably, a combination of the two above classes can be made to produce a shift in the ratio of the effective concentrations of the two naturally occurring cyclic nucleotides. A case in point is in the skin disease, psoriasis. In this condition, it appears that cyclic AMP levels are low while cyclic GMP levels are high (68). To rebalance the ratio, the effective concentrations of cyclic AMP should be increased whereas those for cyclic GMP reduced.

A large number of cyclic nucleotide derivatives have been prepared (73,74). However, the screening methods used to determine their activity were mainly designed to test their ability to mimic the activity of cyclic AMP both *in vivo* and *in vitro*. The ability to antagonize the effects of cyclic AMP or cyclic GMP on specific protein kinases has been largely ignored. There is some evidence, however, that some already known agents and in particular β-adrenergic receptor blockers, *e.g.*, propranolol and sotalol, can act beyond cyclic AMP, *i.e.*, between the formation of cyclic AMP and the physiologic or pharmacologic response (75-79). These agents may be of value in the treatment of juvenile diabetes (80) and hypertension (81). In fact, furosemide may act in this fashion on the toad bladder (82).

It should be noted that compounds possessing the above activity will not only have a great potential as useful drugs but would also provide invaluable tools for the study of the cyclic nucleotide system and its role in biology. Through the use of specific cyclic AMP or cyclic GMP antagonists, the mediator role of cyclic AMP in the action of hormones or drugs and its importance in the regulation of cellular activity could be more convincingly demonstrated.

III. AGENTS AFFECTING THE CYCLIC NUCLEOTIDE SYSTEM SECONDARY TO THEIR EFFECTS ELSEWHERE. Examples of these include:

A. PROSTAGLANDINS, THEIR DERIVATIVES AND ANTAGONISTS: This short review does not intend to cover in detail developments in this rapidly

expanding field. However, it should be mentioned here that prostaglandins are the most powerful modifiers of cyclic nucleotide metabolism known. As a matter of fact, the cyclases, and possibly the phosphodiesterases, appear to be the only enzyme systems affected by these potent lipids in cell-free preparations. Since the prostaglandins appear to be synthesized in and released from cellular membranes, their main function may be to regulate the activity and the sensitivity of the cyclic nucleotide system. As such, they present a good site for the action of compounds designed to modify the latter system. An example of this is the potential utility of anti-inflammatory agents which inhibit prostaglandin synthesis in the treatment of cholera (83), a condition mainly due to increased cyclic AMP synthesis in the intestinal mucosa. Prostaglandin synthesis inhibitors and prostaglandin antagonists may have other applications where increased prostaglandin synthesis occurs. Possible examples include wide-angle glaucoma (84,85), ocular inflammation (86) and excessive platelet aggregation. Already, their enormous potential is being realized in the development of useful drugs in obstetrics, vascular diseases, secretory diseases, skin and possibly cancer as well.

It should also be realized that prostaglandin degradation and synthesis is influenced if not controlled by cyclic AMP (87,88). Modification of the cyclic AMP system would therefore be expected to exert a great influence on prostaglandin activity.

B. AGENTS AFFECTING CATION AVAILABILITY: A situation similar to the prostaglandins also appears to apply in the case of calcium ions (89-91). Ca^{++} generally inhibits AC activity, is essential for GC activity and its efflux appears to be controlled by the prostaglandins (92). Furthermore, activation of protein kinase by cyclic AMP seems to enhance Ca^{++} uptake (93). The interactions between Ca^{++} ions on the one hand and prostaglandins and the cyclic nucleotide system on the other appear to represent the communication complex between living cells and their outside environment. Modification of any

part of this tripartite system affects the other two parts. Mention has already been made of the role played by Mg^{++} in AC activity. Thus modification of the cyclic nucleotide system can be indirectly accomplished via changes of the available Ca^{++} or other cations or the synthesis or activity (*e.g.*, via prostaglandin antagonists) of prostaglandins. This represents another area where modifications of the cyclic nucleotide system can be achieved.

C. OTHERS: The effects of temperature, contact, diet, nervous activity and many other factors are in most cases translated at the cellular level into cyclic nucleotide effects. Modification of any one of these conditions may lead to effective alterations in cyclic nucleotide levels and could be used for the treatment of disease. A possible example of this is the beneficial effects of physical occlusion alone in psoriasis which may be mediated by activation of adenyl cyclase.

It is clear that the cyclic nucleotide system offers numerous opportunities for drug development. To take advantage of these opportunities will require the close cooperation of the synthetic chemists and molecular biologists. Success will require intimate knowledge of the disease-induced abberations in this complex system and the pertinent approaches for their ultimate correction.

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